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By Subjects and Authors

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## INDEX TO ARTICLES

- Abdomen
  - diseases and lesions of 8, 135, 141, 147, 155, 274 281 288, 296, 308, 395 403 533
- Abstracts
  - *January* 131
  - *February* 260
  - *March* 389
  - *April* 520
  - *May* 679
  - *June* 809
- Ambulation
  - during injection repair of hernia 8
- Anesthesia
  - a new form 573
  - in operation for cataract 429
- Aneurysm
  - arteriovenous, of kidney 729
- Anomalies
  - atresia of alimentary tract 147
  - uterus didelphys 422
- Aorta
  - stenosis of 548
- Appendix
  - incomplete removal of 141
- Arteries
  - phrenic, ureteral and gonadal 41
- Arterioles
  - of endometrium 599
- Arteriolization
  - of left ventricle 316
- Arthrodesis
  - of hip 638
- Atresia
  - of alimentary tract 147

- Biliary tract
  - surgical treatment of 288
- Bleeding
  - caused by aspirin 395
  - spontaneous intracerebral 175
- Blood supply
  - variant renal and suprarenal 41
- Bone
  - fractures of 196, 348 359
  - sarcoma of 453
- Book Reviews
  - *January*
    - Harvey, William *Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus (Movement of the Heart and Blood in Animals An Anatomic Study)* 129
    - Popper, Hans, and Schaffner, Fenton *Liver Structure and Function* 130
    - Schroeder, Henry Alfred *Mechanisms of Hypertension with a Consideration of Atherosclerosis* 129
    - Steele, John D , Editor *The Surgical Management of Pulmonary Tuberculosis* 130
  - *February*
    - Alvarez Coca, M , and Urruticoechea, J M *L'Eventration diaphragmatique droite Contribution a son etude (Right Diaphragmatic Eventration A Contribution to its Study)* 257
    - Crowe, Samuel James *Halsted of Johns Hopkins The Man and his Men* 258
    - Cullen, Stuart C *Anesthesia* 257
    - Dripps, Robert D , Eckenhoff, James E and Vandam, Leroy D *Introduction to Anesthesia* 256
    - Hansell, Peter *A System of Ophthalmic Illustration* 259
    - Lippman, Richard W *Urine and Urinary Sediment* 259
    - National Institute of Neoplastic Dis-

eases: *Works in Clinical and Experimental Research* ............ 259
Seward, E. H.: *Inhalation Analgesia In Childbirth* .................... 257
Shippen, Katherine B.: *Men of Medicine* .............................. 258
Thorwald, Jurgen: *The Century of the Surgeon* .................. 256

*March*
Ekman, Carl-Axel: *Portal Hypertension: Diagnosis and Surgical Treatment* .................... 385
Fasanella, R. M.: *Management of Complications in Eye Surgery* ... 385
Martin, Hayes: *Surgery of Head and Neck Tumors* .................... 383
Martius, Heinrich: *Die Geburtshilflichen Operationen, Ihre Ausfuhrung und Anwendungen: ein Lehrbuch fur Studenten und Gebrauchbuch fur Arzte (Obstetric Operations and Technic: A Textbook for Students and Physicians)* ........ 385
[illegible]ini, Paul: *Bases, Principes et Procédés Techniques de la Chirurgie (Fundamental Principles and Technical Procedures in Surgery)* 383
Queno, Jean: *Nouvelle Pratique Chirurgicale Illustrée (New Surgical Technics Illustrated)* ......... 386
Zulch, K. J.: *Brain Tumors* ........ 384

*April*
Bacon, Harry E.: *Chronic Ulcerative Colitis* ........................... 513
Ciba Foundation: *Colloquia on Endocrinology (Hormones in Blood)* ... 516
Congress of Neurological Surgeons: *Clinical Neurosurgery, vol. 3* ..... 516
Cope, Zachary: *The Early Diagnosis of the Acute Abdomen* .......... 519
Dahlin, David C.: *Bone Tumors: General Aspects and an Analysis of [illegible] Cases* .................... 518
Dubost, C., and Blondeau, P.: *Chir[illegible] Open Intra[illegible] Surgery* [illegible] 518
[illegible], Henry C.: *The Gynecologic* [illegible] 517
[illegible] 519
[illegible] 511
[illegible] 515
[illegible] 512
[illegible] 512

Mirizzi, Pablo: *Lithiase de la Voie Biliaire Principale (Lithiasis of the Common Bile Duct)* ......... 515
Pack, George T.: *Tumors of the Soft Somatic Tissues* ........... 514
Pollack, Robert S.: *Tumor Surgery of the Head and Neck* .......... 511
Sjostrom, Bo: *The Activity of Prothrombin and Proconvertin in Plasma in Diseases of the Biliary Tract: A Clinical Study* ......... 513
Van Bogaert, L.: *Cerebral Lipidoses* 512
Werner, Paul and Sederle, Julius: *Abdominal Operations by the Vaginal Route* ..................... 517
Williamson, Martin B.: *The Healing of Wounds* .................... 515

*May*
Cordone, Mario, and Parentela, Aldo: *Hypothermia* .................. 677
Nesbit, Robert E. L. Jr.: *Perinatal Loss in Modern Obstetrics* ...... 678
Puestow, Charles H.: *Surgery of the Biliary Tract, Pancreas and Spleen* 677

*June*
Black, D. A. K.: *Essentials of Fluid Balance* ....................... 807
Chigot, P., and Esteve, P.: *Traumatologic Infantile (The Study of Trauma in Children)* ........... 807
Duhamel, Bernard: *Technique Chirurgicale Infantile (Technic in Pediatric Surgery)* ............. 805
King, Edward L.: *Occipitoposterior Positions* ...................... 808
Meigs, Joe V.: *Progress in Gynecology, Vol. 3* ..................... 807
Nimeh, William: *Symposium on Gastroduodenal Ulcer and Cancer: An International Study* ........... 806
Welch, C. Stuart, and Powers, Samuel R.: *The Essence of Surgery* ..... 806

Breast
  female, carcinoma of ................ 337

Carcinoma
  bilateral bronchogenic .............. 763
  of esophagus ........................ 274
  of female breast ................232, 337
  of female genitalia ................. 337
  of gallbladder ...................... 231
  of nose and accessory sinuses ....... 215
  of prostate ......................... 723
  preclinical, of uterine cervix ....... 92

  of renal pelvis 22
  recurrent, in women 337
  of stomach 135, 274
  of uterine cervix 620

Calculi
  complicating lipoma of corpus callosum 117

Cardia
  gastric, in gastropexy 553

Cardioscopic study
  of aortic stenosis 548

Cardiospasm
  and gastropexy 533

Cardiovascular Surgery
  *February* 160
  *March* 316
  *May* 548
  *June* 706

Cataract
  anesthetics for extraction 449
  extraction as office procedure 429

Cavity
  oral, lesions of 464

Chest
  diseases and injuries of 247, 488, 649

Children
  hemophilic, dental hazards to 641

Closure
  primary, of operative wound 413

Colon
  right portion, granulomas of 683

Complications
  of leg fractures 341

Corpus callosum
  lipoma of 171

Cortex
  cerebral, as affected by SCTZ 573

Decortication
  in total hysterectomy 766

Defibrination 365

Diagnosis
  of cervical carcinoma 92

Disc
  lumbar intervertebral, therapy of 204

Distrubution
  of arterioles in endometrium 599
  of recurrent laryngeal nerve in thyroid disease 649

Diverticulum
  acquired, of male urethra 168

Editorials
  *January*
    Application of Cardiopulmonary Physiology 126
  *February*
    An Appeal to Men of Medicine 252
  *March*
    Indications for Surgical Intervention 380
  *April*
    When the Doctor Goes to Court 504
  *May*
    A Portrait Draped in Black 531
  *June*
    The Pharmacy in History 789

Endarterectomy
  coronary 160

Endometrium
  arterioles of 599

Femur
  fractures of 359

Fibroma
  intrathoracic 655

Fibula
  fractures of 196

Finger
  index, pollicization of 106

Fistula
  vesicovaginal 59, 591

Fracture
  involving legs 348
  multiple 745
  of femoral greater trochanter 359
  of tibia and fistula 196

Fundus
  ocular 629
  uterine 734

Fusion
  of intervertebral bodies 196

Gallbladder
  primary carcinoma of 281

Gastrectomy
  total, for carcinoma 135

Gastroileostomy
  with realignment 155

Gastropexy
  for hiatal hernia and cardiospasm 533

General Surgery
  *January* 1
  *February* 135
  *March* 263
  *April* 395

*y* ... 533
*ne* ... 683
alia
cinoma of ... 337
ourinary Surgery
*uary* ... 22
*bruary* ... 164
*rch* ... 324
*ril* ... 408
*y* ... 533
*ne* ... 683
uloma
right half of colon ... 683

d
read of infection in ... 750
rt
terialization of ventricle ... 316
ophilia
children ... 641
orrhage
sfunctional ... 599
reoperative ... 364
nia
mbulatory injection repair of ... 8
general ... 658
liding hiatal ... 533
rthrodesis of ... 638
rmone
hyroid ... 377
perparathyroidism ... 113
sterectomy
adical vaginal ... 584
tal, with decortication ... 766

itis
[illegible] ... 308
[illegible]
[illegible] of urine ... 740
[illegible]
[illegible] ... 813
[illegible]
[illegible] ... 750
[illegible]
[illegible] ... 196, 318, 359
[illegible] ... 567
[illegible]
[illegible] ... 740
[illegible]
[illegible] ... 280

Kidney
aneurysm of ... 729
infections of ... 567
injuries of ... 567
tumors of ... 22

Laminectomy
for spondylolisthesis ... 101
Lenses
plastic, in anterior chamber ... 629
Leukoplakia
of renal pelvis ... 408
Ligaments
uterine, interposition of ... 740
Lipoma
of corpus callosum ... 171
Lugol's solution
in pregnancy tests ... 179
Lymphadenectomy
for cervical carcinoma ... 620
Lymph nodes
of mediastinum ... 247

Mediastinum
lymph supply of ... 247
Metastasis
extrarectal, from mammary carcinoma 232
of prostatic carcinoma ... 723
Models and moulages
use in teaching ... 1
Mucus
bronchial, postoperative control of.... 485

Neck
infections of ... 750
Nerve
recurrent laryngeal ... 648
trigeminal ... 666
Neuralgia
referable to trigeminal nerve ... 666
Neurologic Surgery
*January* ... 77
*February* ... 171
*May* ... 573
Neuroma
amputation, prevention of ... 77
Newborn
atresia of alimentary tract in ... 147

Obstetric and Gynecologic Surgery
  *January* 92
  *February* 179
  *March* 337
  *April* 422
  *May* 584
  *June* 734

Octogenarian
  surgical treatment of 263

Occlusion
  arterial 706

Operation

Ophthalmologic Surgery
  *April* 429
  *May* 629

Orthopedic Surgery
  *January* 101
  *February* 196
  *March* 348
  *April* 453
  *May* 683
  *June* 745

Osteogenic Sarcoma
  of great toe 453

Otolaryngologic Surgery
  *February* 215
  *April* 464
  *May* 641
  *June* 750

Pedicle
  ovarian, torsion of 194

Pelvis
  renal, tumors of 22

Pharmacy
  in history 789

Phosphate
  colloidal chromic 77

Pin
  double, for fractures 196

Poliomyelitis
  undiagnosed, causing poor posture 460

Pollicization
  of index finger 106

Posture
  poor, resulting from occult poliomyelitis 460

Proctologic Surgery
  *February* 322

Prolapse
  uterine and vaginal 186

Prostatectomy
  retropubic 324
  suprapubic 413

Protein
  serum level of 563

Repair
  of hernia 8, 403, 533
  of nasal septum 757

Resection
  submucous 757

Rhinitis
  atrophic 472

Sarcoma
  osteogenic 453

Seccão em Portuguez 774

Seccion en Español
  *March* 3[illegible]
  *April* 496
  *May* 666
  *June* 779

Section en Français
  *March* 364
  *May* 658
  *June* 766

Sinuses
  nasal and accessory 215

Splenoportography 288

Spondylolisthesis
  treated by laminectomy 101

Stenosis
  aortic 548

Stomach
  glomus tumors of 699
  resection of 135, 274, 533

Surgery of the Thyroid and Parathyroids
  *May* 649

Survival rate
  of prostatic carcinoma 723

Syndrome
  Horner's, associated with fibroma 655

Tendons
  interdigital, tumors of ............. 774
Tests
  for pregnancy ..................... 179
Thoracic Surgery
  *February* ........................ 247
  *April* ........................... 485
  *May* ............................. 649
  *June* ............................ 763
Thyroid
  diseases of ....................... 649
Tomography .......................... 496
Torsion
  of ovarian pedicle ................ 144
Transplant
  autogenous ovarian ................ 734
T tube
  surgical use of ................... 274
Tuberculosis
  urogenital ........................ 563
Tuboplasty
  with polyethylene ................. 368
Tumor
  of breast ......................... 337
  of gallbladder .................... 281
  of genitalia ...................... 337
  glomus, of stomach ................ 699
  of kidney ......................... 22
  of nose and sinuses ............... 215
  of prostate ....................... 723
  of stomach ............... 135, 274, 649
  of uterine cervix ................. 620

Tympanoplasty
  transmeatal and endaural .......... 221

Urethra
  male, diverticulum of ............. 168
Uterus
  carcinoma of .................. 42, 620
  double ............................ 422

Vagina
  as approach to hysterectomy ....... 584
Varicocele
  treatment of ...................... 164
Vein
  varicose, treatment of ............ 719
Ventricle
  left cardiac, arterialization of .. 316
Visualization
  direct, in aortic stenosis ........ 548

## INDEX TO AUTHORS

Abbott, Kenneth G. .................. 204
Antoine, T. ......................... 620
Ayral, M. Naci ...................... 655

Bacon, Harry E. ..................... 232
Baehr, Frank H. ................ S, 403
Bailey, Charles P. .................. 160
Barbosa, Edgard ..................... 281
Barnes, Neale ....................... 485
Barraquer, Joaquin .................. 629
Bastiaanse, M. A. van B. ............ 591
Beaconsfield, Peter ................. 263
Beck, Ernst ......................... 247
Bell, Houston J. .................... 621
Beltrame, Enrico .................... 164
Bernstein, David .................... 757
Boerema, I. ................... 135, 533
Boshamer, K. ........................ 563
Buckles, Maurice G. ................. 126
Bunnag, Tawan S. .................... 296

Carron, C. .......................... 766
Cascino, Joseph P. .................. 171
Chareton, L. ........................ 573
Charronet, R. ....................... 573
Ching, Renald ....................... 429
Christensen, Howard W. .............. 331
Cohen, Herbert G. ................... 196
Coirault, R. ........................ 573
Coqui, Carlos ....................... 496
Cottle, Maurice H. .................. 472
Dailey, Ulysses G. .................. 308

D'Alonzo, Walter A. .................... 683
Damasio, R. .................... 573
Daneshvari, Mostafa .................... 126
Dena, Feliziano Palomino .................... 779
Dennison, Wallace K. .................... 644
De Weerd, James H. .................... 567
Donovan, Robert J. .................... 699
Douglas, George C. .................... 734
Duchesne, Ronald .................... 316
Duran, Rodolfo .................... 274

Esnaurrizar, Miguel Lopez .................... 666

Fabrizy, P. .................... 573
Fetter, Theodore R. .................... 22
Figi, Frederick A. .................... 215
Fontoura, Hamilton .................... 531

Gaujard, R. .................... 573
Graham, James H. .................... 699
Greene, Earle I. .................... 141
Greene, J. Major .................... 141
Griffenhagen, George .................... 789
Gürkan, Kazim Ismail .................... 763

Haas, Alfred .................... 155
Hattori, J. .................... 548
Hellwig, C. A. .................... 408
Hendelman, M. H. .................... 194
Hill, Robert M. .................... 422
Hoffman, Howard A. .................... 729
Högler, Hans .................... 584
Hunt, Claude J. .................... 288
Hunt, William T. Jr. .................... 453

Iikawa, T. .................... 548
Inomata, K. .................... 548

Jackson, Charles .................... 171
Jacobson, Herman A. .................... 263

Kaplan, Arnold .................... 171
Karakashian, N. A. .................... 449
Karafin, Lester .................... 723
Kasdon, S. Charles .................... 740
Kosyak, Jenya .................... 274
Kraft, George L. .................... 348

Laborit, C. .................... 573
Laborit, H. .................... 573
Lake, Frederic .................... 171
Lange, Max .................... 638
Lamphier, Timothy A. ....................106, 395
Lechat, P. .................... 573
Lemieux, J. Edouard .................... 655
Leon, Lucina Villegas .................... 779
Levy, Marvin S. .................... 113
Levinthal, Daniel H. .................... 348
Lichtenstein, Irving L. .................... 113
Loeser, Alfred Alexander .................... 337
Lopez V., Esteban Alfonso .................... 374

Marks, Mark M. .................... 1
May, Angelo M. .................... 160
McCrea, Lowrain .................... 728
McCusker, E. .................... 408
McPheeters, H. O. .................... 719
Merklin, Robert J. .................... 41
Meyer, Karl A. .................... 263
Michels, Nicholas A. .................... 11
Miller, C. O. .................... 16
Misischia, Nicholas .................... 17
Mower, Jack Henry .................... 1
Murphy, J. Peter .................... 17
Myers, T. B. .................... 232

Nery, Robert .................... 274

O'Donnell, Anne R. .................... 699

Peckler, David ....................141
Pellet, C. .................... 361
Pernet, Alipio .................... 771
Perl, John I. .................... 77
Pinto, V. Carvalho .................... 147

Rate, Robert G. .................... 281
Ratzan, Martyn C. .................... 359
Ritter, Saul Alfred .................... 155
Roll, W. A. .................... 408
Rowe, Daniel H. .................... 731
Rowe, Robert B. .................... 413
Rubenstein, Alan .................... 496
Rubio L., Boris .................... 468

Sakakibara, S. .................... 518
Salvatore, Carlos A. .................... 599

Savastano, A. A. 453
Schönbauer, L. 649
Schumer, William 141
Segraves, James E. 745
Seletz, Emil 504
Soule, Edward H. 92
Stuteville, Orion H. 464, 750

Thom, Charles H. 186
Thomson, J. C. 644
Thorek, Philip 274
Totten, H. P. 706

Vernon, Sidney 380
Villalba Lopez, G. 232
Vineberg, Arthur 316

Waller, J. D. 408
Weinstock, Horace L. 324
Whisler, Edna 449
White, Malvin F. 106
Whitlock, David G. 77
Wilkerson, J. Louis 22
Willner, Philip 460
Wilson, Milton J. 194
Wiltberger, Ben R. 204
Wolfe, Henry E. Jr. 413
Woolsey, Robert Dean 101

Young, W. Arthur 395

Zeitlin, A. B. 168

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## General Surgery

# Models and Moulages: Their Use in Teaching

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RECENT advances in the development of synthetic materials with plastic properties have stimulated interest in model making. In the past, models used in medical teaching have been constructed of wax, papier mache or plaster of paris. Those produced in quantities by mass production methods were often poorly done. The better ones required a high degree of artistic skill and much time to produce; this made them expensive and rare. Some were subject to deterioration from aging and changes in temperature. Others, because of their fragility, were usually relegated to a glass case for observation and became unavailable for close examination.

For these reasons, models have not been adequately employed in basic science classes or in medical schools. Good models can be particularly helpful in such subjects as embryology and comparative anatomy. Microscopic objects enlarged in models will show greater detail. Models can be of inestimable value in the study of anatomy, as exhibits to illustrate special operative measures, and in the recording of actual surgical observations at operation or at autopsy. Other subject matter for model and moulage making can be obtained from a variety of sources, e.g., one's own knowledge or drawings or photographs in medical texts.

It is my purpose in this paper to describe relatively simple methods for making models and moulages that are particularly suitable for medical teaching. They

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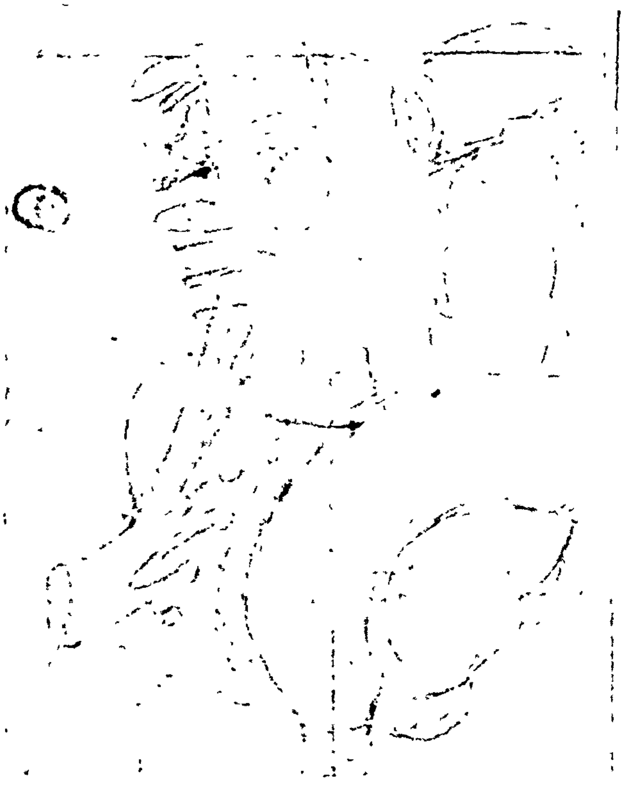

Fig. 1.—Freehand drawing on plyboard.

are made of readily available materials, require little skill to make, and can be handled without fear of breakage. Their accuracy is good and their cost small.

Five basic steps are required in making these models: (a) drawing, (b) modeling, (c) making the mold, (d) pouring the positive solution and (e) coloring. The form to be fashioned is first drawn with a pencil on a piece of thin plywood 4 inches (10 cm.) larger than the form itself. Enlargements of drawings from other sources are made by multiplying the size of squares. If the original drawing is lined with half-inch squares, the plywood is covered with 1 inch squares to double the dimension or multiples of ½ inch for the desired increase. A freehand drawing is usually sufficient, since the penciled lines are used only as a guide (Fig. 1). When the sketch is completed, plastiline or oil clay is placed to conform to the drawn figure and roughly shaped to make a bas relief (Fig. 2). The use of a sculpturing tool defines detailed formations more clearly; care should be taken not to create overhanging clay or lipping that could interfere with removal of the cast.

Investment material may be plaster of paris, ceramic plaster or commercial molding plaster such as is used in construction work. To make liquid plaster for taking impressions, a quantity of water estimated to cover the clay to a depth of ½ inch is first put into a mixing bowl. Dry plaster is rapidly sprinkled into the water until the latter will absorb no more fluid. Not until then should the mixture be stirred by hand or with a spatula until it is smooth. To remove air bubbles that may interfere with reproduction, the container with the liquid plaster should be gently bounced. When the plaster begins to thicken slightly, it is slowly poured over the modeled clay until the latter is evenly and completely covered. If the piece is larger than a foot square, gauze or cheesecloth incorporated into the mold at this stage will strengthen it and prevent breaking. Should weak or thin spots in the mold become apparent or be suspected, more plaster and water can be mixed and added to make the entire cast ½ to ¾ inch thick.

When the cast becomes warm, it has hardened sufficiently to be safely moved for separation from the clay. Depressing the edges of the plywood board away from the plaster will loosen the cast so that it can be lifted free from the clay model. If separation is difficult and some clay adheres to the plaster, it can be removed with a wooden applicator stick. This will prevent damage to the negative and interference with good reproduction. While the plaster is still moist, additional carving with a sharp tool may be done if required. The plaster negative is dried at room temperature (Fig. 3). If a quick drying is desired, it may be slowly warmed in an oven.

The positive solution consists of three parts, clay, water and latex. The last-mentioned substance is a synthetic, water-soluble rubber. The purpose of this mixture is to make a rubber-based slip or thick liquid which, when put into a dry plaster mold that extracts the water, builds up a clay-rubber layer giving the exact impression of a positive form. The proportions of the component parts of the positive solution are water, 25 per cent; McNamara clay, 25 per cent, and anode latex, 50 per cent. The clay is water-washed and is high in kaolin, and is made up of exceedingly fine particles. The water and clay are mixed before being added to the latex. In this combination the ingredients form a thick, viscous liquid. The solution may be obtained in this formula from the Latex Corporation of Chicago or may be purchased separately from ceramic or chemical establishments. If the mixture is produced by the model maker, 20 drops of sodium silicate added to 1 pint of the latex-clay mixture will act as a deflocculent and keep it from separating.

The plaster mold should be bone-dry before it is used. To make the positive impression it is best to level it and make it secure to receive the liquid. Care must be exercised to prevent spillage, because of the latex-clay mixture's adherent properties in the fluid state. The cavity of the plaster negative is filled slowly to prevent air pocketing and splashing. When full to capacity, the cast is left undisturbed for thirty to forty minutes or until a clay-latex wall 1/8 inch thick has accumulated. The remaining liquid is returned to airtight container and stored in a cool place for future use.

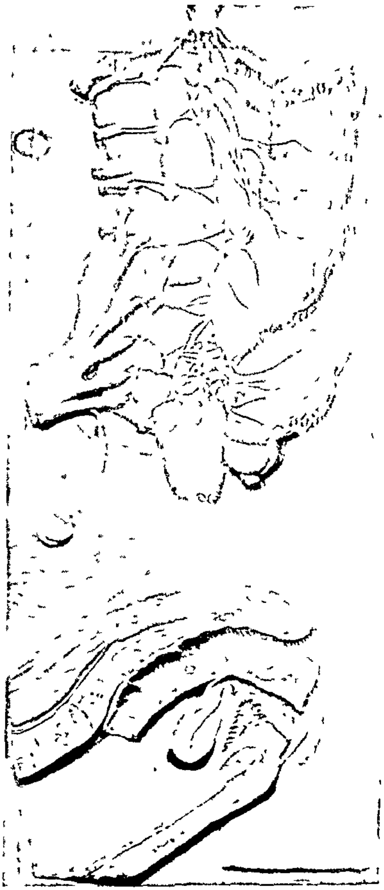

Fig. 2—*Above*, clay model ready for casting *Below*, model of section through female pelvis in knee-chest position

Impressions made in this way need backing to stiffen them and help maintain their shape. To do this the remaining portion of the cavity in the mold is filled with crumpled paper, gauze or mechanic's cotton waste. Some of the positive solution can be used as a binding material to anchor the backing to the impression itself for greater stability. The completed form is set aside at room temperature for twenty-four to seventy-two hours, depending upon the size. This allows for air-curing of the clay-latex in the cast. After the model has been removed from the mold, it is dried again for another twelve hours. There will be about 5 per cent shrinkage. The form is then ready for coloring. I find that pigments in casein and in acrylic polymers in latex give the models a more realistic coloration. Both may be obtained in an art shop. The advantage of these over other types of colors is that they are permanent, dry rapidly and remain flexible for longer usage. The accuracy of coloration is dependent upon the care and diligence of the model maker (Fig. 4).

Moulages are molds or casts taken directly from a portion of the body. Such impressions obviate the need for modeling. ace masks or impressions of skin lesions re examples of this. When this procedure is combined with sculpturing in plastiline, however, many possibilities become evident.

Fig. 3.—Plaster cast of clay model.

When a body surface impression is taken, the cavity, or negative, in the plaster can be carefully packed with clay to return the original surface. By dissecting the clay and modeling the structures beneath, the anatomic character of the parts can be demonstrated. As further plaster impressions are made of the sculptured parts of the dissection, a series of moulage-models can be easily produced to show structures at various levels.

To illustrate such a project, suppose a thyroidectomy is to be shown. A plaster impression is taken of the neck and upper part of the cadaver. When the plaster hardens it is removed. Clay is carefully pressed into the cavity to pick up skin texture and body shape. When the plaster is removed, the clay, simulating the original form, is ready for modeling. The line of incision is made in the clay as it would be in the skin. A plaster impression is made of it, along with the surrounding area. As the skin flaps are raised and other structures normally encountered are modeled, impressions are taken at each important step and removed until the series is complete. When all of the plaster casts are dry, the cavities are filled with the positive solution and the moulages finished as previously described. To simulate naturalness still further, the dissected parts are painted with a clear plastic glaze after coloration, to heighten the colors and produce highlights that give the illusion of moisture.

The same procedure can be followed to portray and illustrate such things as axillary, perineal, neck, and other dissections; the Glover operation for coronary artery disease; displaced intravertebral discs; hernias; amputations; plastic or orthopedic procedures and, for that matter, nearly all operations that can be doc-

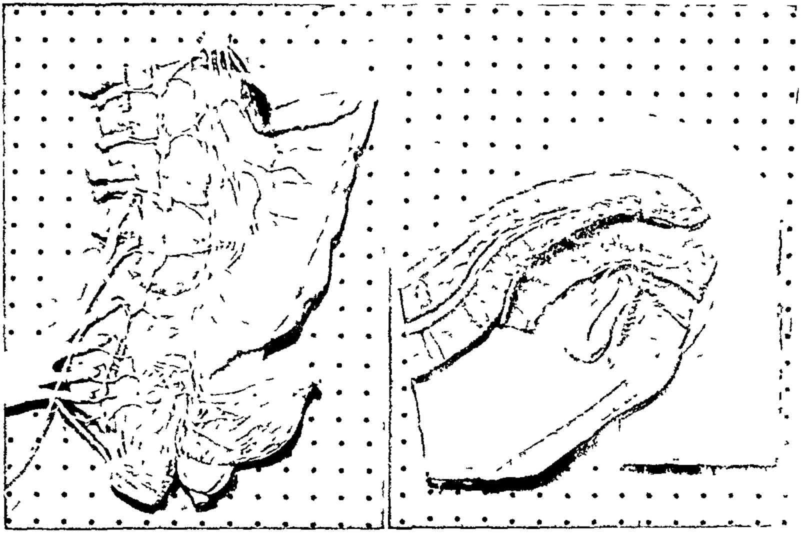

Fig. 4.—Models completed, showing innervation of left half of colon and female figure

umented for teaching, reference or permanent record.

Bones can be accurately reproduced by casting them in multiple piece molds and using a synthetic resin in a liquid state that hardens into a horny material when dry (Fig. 6). Other synthetic resins, such as styrene chloride, are suitable for smaller model and moulage making. This and other like substances require maturing at 375 F. for fifteen minutes in metal molds.

Another potentiality of model and moulage making that should be explored is what it can do for the student. It has been shown that those who learn the process through a specific project of their own gain much more from the subject. The better specimens made by these students can be used to teach following classes. Most students, through this experience, find a new medium for the training.

I have found these models useful in a office practice, for in my experience most patients are vitally interested in the nature of their ailments and not only are grate but become more cooperative when t physician takes them into his confiden In many instances a careful explanation with such an illustration removes much of the unnecessary mystery and establishes greater confidence. Models and moulages such as I have described have been employed for this purpose with good effect.

On television and in medical motion pictures models and moulages of this type have been extremely useful, because of their realism, in illustrating spec a- tomic problems and orienting su cedures.

Fig. 5.—Sagittal section through anterior rectal wall, prostate and bladder.

Fig. 6.—Reproduction of pelvis.

CONCLUSION

Because of the magnitude of medical teaching, any means that can accelerate the student's learning is worth while. Previous methods for making useful three-dimensional models and moulages have seemed formidable to those who could use them for instruction. A method of making such models and moulages is described that affords access to endless possibilities as new materials are developed. Their uses are explained in detail.

RÉSUMÉ

Etant donné le domaine très vaste des études médicales, tous les moyens susceptibles de les faciliter et de les accélérer doivent être pris en considération. Certaines méthodes permettant de fabriquer des modèles et moulages à trois dimensions ont été accueillies avec enthousiasme par les professeurs. L'auteur décrit une méthode inédite donnant des possibilités infinies grâces à des matières nouvelles.

CONCLUSAO

Devido a magnitude e importancia do ensino médico qualquer meio de treinamento dos estudantes deve ser considerado importante. Os métodos anteriores utilisando modelos e modelagens tridimensionaistem sido formidaveis para os que os utilisam como elementos de ensino. O A. descreve a técnica de faser esses modelos e modelagens, usando recursos para utilisar ao máximo as possibilidades dos novos materiais. Explica o uso pormenorisadamente.

SCHLUSSFOLGERUNGEN

Angesichts des Umfanges des Materials des medizinischen Unterrichts ist jedes Mittel, das dem Studenten ein schnelleres Lernen ermöglicht, zu begrüssen. Frühere Methoden der Herstellung nützlicher dreidimensionaler Modelle und Moulagen scheinen die Lehrer, die solche Hilfsmittel gebraucht hätten, von ihrer Anwendung abgeschreckt zu haben. Es wird ein Verfahren zur Anfertigung solcher Modelle und Moulagen beschrieben, das infolge der Entwicklung neuer Materialien unabsehbare Möglichkeiten eröffnet. Ihre Anwendung wird im einzelnen dargestellt.

### CONCLUSIONI

La vastità dell'insegmento medico rende degno di considerazione ogni mezzo che consenta di affrettare la preparazione degli studenti di medicina. L'impiego, ad esempio, di calchi e modelli a tre dimesioni si è dimostrato di incalcolabile utilità.

L'autore descrive appunto un metodo per costruire tali modelli e per usarli con profitto.

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# The Ambulatory Injection Repair of Inguinal Hernias

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SPRINGFIELD, MASSACHUSETTS

AN ambulatory injection treatment for male inguinal hernias that produces, by piezoelectricity, a nonabsorbent granulomatous tissue that acts as a permanent internal truss beneath the external oblique aponeurosis of the groin will be described. This procedure is based upon twenty years' practical experience in selected cases. An evaluation of the end results of all inguinal hernias treated in a three-year period with an eight- to ten-year follow-up shows that the technic described overcomes the problems encountered with earlier methods. In order to obtain statistical information on which to base a conclusion with respect to results, 214 male patients with 261 inguinal hernias treated in the office, with the treatment completed during the years 1946, 1947 and 1948, were followed up from September 1955 to September 1956.

Experience has shown that two factors are essential to success with the ambulatory treatment: (1) a sclerosing solution that produces a nonabsorbent tissue in the affected area of the groin, and (2) a reliable truss to support the patient completely and without discomfort throughout the treatment.

The means of evaluating results, experiences, observations and the underlying causes of failure will be discussed.

*History of the Injection Treatment.*—As far back as 1880, so noted a surgeon as Billroth[1] *stated that radical operation for* hernias could be done away with if artificial proliferation of tissues could be produced. About 1900, Mayer[2] of Detroit *modernized the ambulatory treatment* originated by Velpeau[3] in 1835. During the 1930's Bratrud and others[4] reported excellent initial results with the injection treatment in large series of cases. Biopsies performed by Kelly[5] on injected animals at ten-day intervals, however, showed that early fibroplastic tissue had practically disappeared in one hundred days and had been largely absorbed within fifty days. Dobson,[5] in 1940, confirmed Kelly's experiments. Coley,[5] of the Hospital for the Ruptured and Crippled in New York City, observed 81 per cent of relapses after injection treatments. At this time the Council of the American Medical Association, in its official Journal,[6] stated: "After due consideration of a second hospital survey the *Council reaffirms its previous opinion that* the Injection Treatment may not be recognized for general use. Universal employment of the method is to be deplored." Since that period there has been a dearth of scientific articles on this subject.

In 1949, however, Koontz[7] stated, "The *injection method has a place in the treatment of hernia, especially for patients* coming in the following categories: (a) elderly persons unsuitable for operation *due to the infirmities of old age*; (b) persons rejected for industrial employment because of enlarged rings, with doubtfully demonstrable hernias; (c) patients who will not or cannot be operated on for personal reasons; (d) persons with certain types of recurrent hernias with small fascial defects." To this may be added pa-

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tients who, for any reason, cannot absent themselves from their jobs for any length of time.

*The Sclerosing Solution.*—Several different mixtures of irritating chemicals, manufactured by well-known pharmaceutical concerns, were tried prior to 1944 These irritating chemicals produced fibrous tissue when introduced into the inguinal canal, but the areas of injection had to be well overbuilt to allow for natural resorption of the irritant, which often occurred after the procedure had been completed. This resorption varied in amount in different patients, but seemed greatest in men over 50 years of age. Often the patients so treated would require extra injections after their original series had been completed, in order to prevent a recurrence. No doubt this resorption, with consequent opening of the canal, was the main reason why many surgeons discontinued the ambulant injection treatment It became evident that a new sclerosing solution was needed to form a permanent tissue.

A search through the medical literature disclosed the discovery made by McNally and Bergman[8] during their investigation of silicosis in 1935. These two men abandoned the theory that the hardness and sharpness of silicon particles alone produced the chronic pathologic conditions and strange fibrous tissue associated with silicosis of the lungs. They decided that silicosis is caused by the action of the blood fluorides on the silicon molecule, forming soluble tetrafluoride and thus producing fibrotic changes. The theory that fibrosis of the lungs is caused by the chemical action of sodium fluoride in the blood upon silicon dioxide was novel. To test it out, these investigators made up a near-colloidal solution of calcium fluoride containing finely suspended particles of silicon dioxide or quartz and injected it into animals. These injections produced nonabsorbent fibroplastic tissue similar to that observed in cases of silicosis. This discovery prompted McNally and Bergman[8] to advocate the use of such a solution for the injection treatment of inguinal hernias The solution was called Alparene No 2*

*The Piezoelectric Theory.*—Evans[9] in 1948 and Evans and Zeit[10] in 1949 demonstrated that when silicon dioxide (quartz) is deposited in living tissues a fibrogenic effect is produced. The silicon dioxide (quartz) crystals belong to a class that lacks a central point of symmetry, they are therefore piezoelectric These authors defined "piezoelectric" as that property of asymmetric crystals that causes them to develop electrical charges when compressed or overextended in particular directions Cough, strain, muscular activity, arterial pulsation or any physiologic mechanism that can bring about a squeeze [illegible] the piezoelectric crystals deposited [illegible] the tissues is capable of producing a current of electricity. Kaplan[11] in 195[illegible] added mechanical pressure from restricting garments and trusses These asymmetric crystals are observed to be fibrogenic when introduced as a finely divided dust into living tissue, uniformly producing proliferative fibrous reactions. Evans and Zeit[10] postulated that crystalline materials are fibroplastic if they are relatively insoluble in body fluids, are in the asymmetric (piezoelectric) crystal group, and are electrically nonconductive, of a proper particle size and in sufficient particle concentration. Alparene No. 2 is in this class

In 1953, Kaplan[11] removed nodular masses that had been formed after Alparene No. 2 had been injected into the groins of 4 patients who had been treated by the ambulant method.

Examination revealed that the reaction of tissue to silaceous material appeared to be primarily fibroplastic, with little or no inflammatory response. Investigations to

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*Dequin Physicians Products Co. Chicago

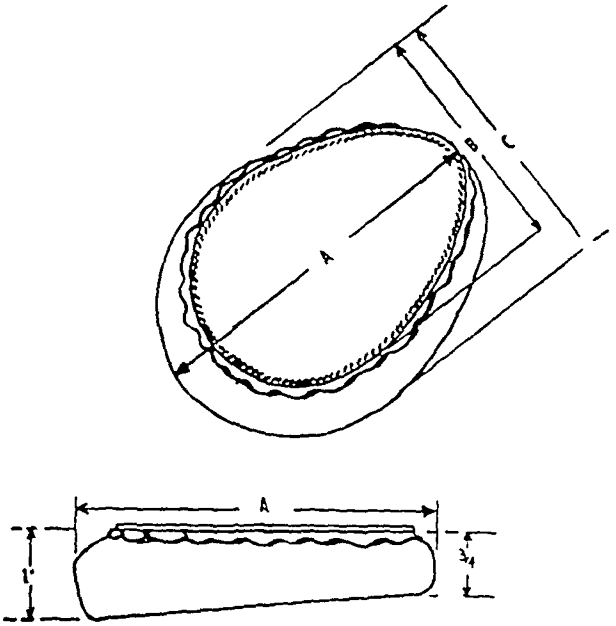

Fig. 1.—Inguinal pad. In upper drawing line *A* is 4 inches (10 cm.) in actual length; line *B* is 2½ inches (6.2 cm.) long, and line *C* measures 3 inches (7.5 cm.). Lower drawing (view showing depth of pad) repeats line *A*. Pad is 1 inch (2.5 cm.) thick at lower end (shown on left) and ¾ inch (1.9 cm.) thick at upper end (shown on right).

determine the factors responsible for this type of tissue response revealed early that the concentration of silica and its particle size determined the development of the nodular fibrosis characteristic of this material.

On the basis of the work of Evans and Zeit[10] and Kaplan[11] one may assume that it is the piezoelectric process which causes the pronounced fibrogenesis.

*Support During Treatment.*—Two trusses are required to be worn by the patient: (1) a semirigid truss for day wear and (2) an elastic one to be worn in bed. The semirigid truss keeps an even support of the groin, even under pressure. The framework of the semirigid truss encircles two thirds of the body, and elastic webbing encircles the other third. An ovoid inguinal pad (Fig. 1), thicker at the medial end than the lateral, and entirely covering the involved groin, is applied just lateral to the base of the penis. The covering of the external ring and the lateral part of the pubic bone is most essential, as the first four to six injection treatments completely close the external ring and apparently fuse this area with the pubic bone. A perineal leg strap without any tension is added to the framework (Fig. 2).

The truss made entirely of elastic webbing, with the same type of ovoid inguinal pad, is used for bed wear. (The construction and application of these trusses will be discussed in a later article.)

*Selection of Patients.*—Male patients who can be successfully treated are those with small to medium sized inguinal hernias, either direct or indirect or a combination of both, with good musculature that can be comfortably and completely held at all times by a properly fitted truss, and in whom the external ring is not greater than 3 cm. in diameter.[12]

The exceptions are patients who have a coexisting chronic cough, are obese, have large varicoceles, or have severe constipation or urinary obstruction caused by a hypertrophic prostate. The injection therapy has not been successful for women, owing to anatomic differences.

*The Role of the Patient.*—The patient must be conscientious and cooperative to insure maximum success with the injection procedure. He must wear the semirigid day truss while up and about and the elastic night truss with the same type of

*Recurrent Hernias*

| No. of Patients | No. of Hernias | Recurrences within 2 Years | Recurrences from 2-10 Years | Total Recurrences |
|---|---|---|---|---|
| 205 | 249 | 13.7%<br>34 cases | 10.8%<br>27 cases | 24.5%<br>61 cases |

elongated ovoid pad while in bed The night truss must be worn for two months during the initial part of the treatment As a precautionary measure during this period, the patient must change from his day to his night truss while lying on his back No tub baths are allowed until after the first four to six injection treatments At the end of this period (four to six weeks) the external ring should be completely and tightly closed and the patient may take tub baths without support Prior to this time, sponge or shower baths are prescribed and are taken in the morning with the night truss on After the bath the elastic truss is wrung out and hung up to dry in readiness for wear again that evening

*Office Requirements* —The following equipment is needed for office treatment

1 A sterile short beveled 3 inch No 18 bore needle attached to a 5 cc Luer Lok syringe

2 Gauze pads and alcohol

3 A wide mouthed bottle of oil of sesame with 3 per cent phenol

4 A 60 cc rubber-capped vial of Alparene No 2 (Alparene No 2 consists of calcium fluoride and silicon dioxide in the form of a "near colloidal' suspension containing 2 5 Gm of each per 30 cubic centimeters of solution )

*Treatments and Doses Required* —The reactions to Alparene No 2 vary with the individual patient With experience, I have learned that the dosage can be kept at a level at which minimal soreness and swelling are involved

The average patient may or may not have some soreness and swelling after the first three or four injections If he does, it usually is not severe enough to keep him from carrying out his daily routine

The suggested doses are

First treatment 1 cc of Alparene No 2 diluted and mixed well with 3 cc of oil of sesame with 3 per cent phenol

Second treatment 2 cc of Alparene No 2 with 3 cc of oil of sesame with 3 per cent phenol

Third treatment 1 cc of Alparene No 2

Fourth treatment 2 cc of Alparene No 2

Fifth to tenth treatments Usually 2 cc of Alparene No 2 given once or twice weekly if no soreness exists

Subsequent treatments 2 cc of Alparene No 2

For very muscular patients doses beyond the tenth may consist of 3 cc of Alparene No 2 Very thin patients usually require a lesser number of treatments and smaller doses

To the average well developed male patient the first four to six injections are given at weekly intervals Each one is made just inferior in position to the preceding one, taking in the width of the inguinal canal Some patients who have complained of a "dragged-down feeling" find that this disappears after three to four injections As a rule, these four to six injections completely close the external ring and no protrusions or impulses can be felt when the patient coughs or strains Closing the external and internal rings tightly does not permanently interfere with the blood supply of the testes The medial third of the canal will now be quite solid and will seem fused to the pubic bone, and Hesselbach's triangle will be reinforced, which can prevent thinning out of this area in middle life This apparent localizing of the solution in the medial third of the canal is probably due to man's upright position, which forces more of the chemical to be deposited near the pubic bone and therefore causes hardness in this area first Subsequent injections, averaging ten to fifteen more, are made in the same pattern, but usually only in the lateral two thirds of the canal and posterior to the newly formed tissue Invagination of the scrotum with the left index finger is done prior to these injections only to determine the location of the closed external ring, which aids in determining the direction of the needle Palpation of the injected areas of injection may reveal small spots that are

*Technic for Recurrent Surgical Hernias.*—The majority of surgically recurrent inguinal hernias become direct hernias. Observation and experience have shown that some so-called recurrent surgical hernias are nothing more than concomitant direct inguinal hernias that were overlooked during the primary operation. These recurrent hernias are treated by injecting the canal, particularly the lower third or the lower half. Surgically recurrent inguinal hernias, consisting of small localized fascial holes that are usually due to a suture cutting through the tissues or to a localized infection, are treated by depositing the chemical, without pooling, beneath the external oblique fascia into these areas. The granulomatous tissue then fills in the defects. Usually fewer treatments are used for this type of inguinal hernia.

*Technic for Potential Hernias:* The potential inguinal hernia, always an industrial problem, can be permanently relieved and the enlarged rings completely closed with a few injections. The patient will then be free from impulses and acceptable for employment.

*Group Studies.*—The original diagnoses in the cases of 214 patients aged 15 to 79, with 261 inguinal hernias (47 being bilateral), were as follows:

| | |
|---|---|
| Right indirect | 18 |
| Left indirect | 14 |
| Right direct indirect | 97 |
| Left direct indirect | 85 |
| Left indirect scrotal | 3 |
| Left direct indirect scrotal | 1 |
| Right recurrent surgical | 13 |
| Left recurrent surgical | 12 |
| Right recurrent injection | 5 |
| Left recurrent injection | 3 |
| Right recurrent injection done elsewhere | 1 |
| Right recurrent surgical 2 previous herniotomies | 6 |
| Left recurrent surgical 2 previous herniotomies | 2 |
| Left recurrent surgical and injection done elsewhere | 1 |
| | 261 |

It is realized that the diagnosis of indirect versus direct hernia by finger palpation has a large margin of error. The true diagnosis often can be proved only by operation.

The causes given by the patients for these hernias are as follows:

| | |
|---|---|
| Lifting | 129 |
| Unknown | 94 |
| Accident | 35 |
| Coughing | 3 |
| | 261 |

We were unable to trace 9 patients with 12 inguinal hernias. In this group there were 5 right and 1 left direct indirect, 3 left and 1 right indirect, 1 right and 1 left recurrent surgical.

Thus, the final number to be discussed is 205 patients with 249 inguinal hernias in whose cases there were 61 recurrences, listed as follows:

51 patients with 54 recurrent inguinal hernias
5 deceased patients with 5 recurrent hernias
2 patients sent questionnaires with 2 recurrent hernias

There were 22 patients with 26 hernias who died of intercurrent diseases. All but 6 of these patients were over 50 years of age. With the aid of their attending physicians and surgeons and the hospital records, I was able to learn the causes of death and listed them as follows: 15, cardiac disease; 4, carcinoma (2 pulmonary, 1 gastric and 1 rectal); 1, sarcoma of the neck; 1, cirrhosis of the liver, and 1, cerebral hemorrhage. Of the 26 hernias, there were no recurrences in 22, but there was 1 enlarged ring; there were 3 definite recurrences; and there was 1 questionable recurrence in a patient who was dead on arrival at a local hospital.

The 22 nonrecurrent inguinal hernias in the deceased patients are listed as follows as to the approximate number of years they were without recurrence before death:

| | | |
|---|---|---|
| 3 years | 3 | |
| 4 years | 3 | |
| 5 years | 1 | |
| 6 years | 5 | |
| 7 years | 5 | (1 enlarged ring) |
| 8 years | 4 | |
| 9 years | 1 | |

Those with recurrences were:

| | |
|---|---|
| 2 years | 1 |
| 6 years | 1 |
| ? years | 2 |

One hundred and sixty patients with 192 inguinal hernias were examined by other physicians and surgeons before coming to the office to receive the ambulant treatment. Of this number, 82 patients with 98 hernias were "insurance cases," and their fees for the injection therapy were paid for by 19 different insurance companies and 5 factories. Also, there were 4 employees (with 6 hernias) from the City of Springfield, which assumed responsibility for the cost of the treatments. All of the 160 patients covered by insurance came of their own volition.

All patients were examined personally except 5, to whom questionnaires were sent. These 5 patients had 6 hernias (1 right, 3 left direct indirect and 1 bilateral direct indirect). Four had no recurrences after ten years; 2 cases had recurrences, 1 after three years, and 1 patient did not know how many years he had had his recurrence, as he was symptom-free.

In the age group over 50 there were 86 patients with 103 inguinal hernias; i.e., 41.9 per cent of the patients had 40.9 per cent of these hernias. In the same age group, 20 patients (including those deceased) had 21 recurrent hernias; i.e., 23.2 per cent of these patients had a recurrent rate of 20.3 per cent.

There were 30 patients with 33 inguinal hernias who were seen after they had had a recurrence of a surgically repaired hernia. Twenty-five of these recurrent surgical hernias (including those of deceased patients) were without recurrences following the ambulant injection therapy after the following number of years:

| | |
|---|---|
| 3 years | 2 |
| 4 years | 1 |
| 6 years | 2 |
| 7 years | 3 |
| 8 years | 5 |
| 9 years | 5 |
| 10 years | 7 |

I was unable to trace 2 of these patients.

After the injection therapy, 6 patients with recurrent surgical hernias again had recurrences after the following number of years:

| | |
|---|---|
| 1 year | 1 |
| 3 years | 1 |
| 10 years | 2 |
| ? years | 2 |

Seven patients with 9 hernias, 6 of whom were treated at my office and 1 of whom was treated elsewhere prior to 1946 had recurrent injection hernias. The solution used was not Alparene No. 2. After the series of treatments with Alparene No. 2 there was 1 recurrence after seven years. Eight hernias were without recurrences after the following number of years:

| | |
|---|---|
| 8 years | 2 |
| 9 years | 1 |
| 10 years | 5 |

There was a recurrence in a patient who had undergone both operation and injection therapy, which was given elsewhere, and who was treated at my office in 1946. He had no recurrence after ten years.

In this series, 5 patients had had primary hernias (1 right indirect, 2 right and 2 left direct indirect) previously treated by injection in my office in the years 1937, 1938, 1942, 1943 and 1944 and were without recurrences. In 1946-48 they returned for the ambulant treatment of newly developed inguinal hernias in the opposite groin.

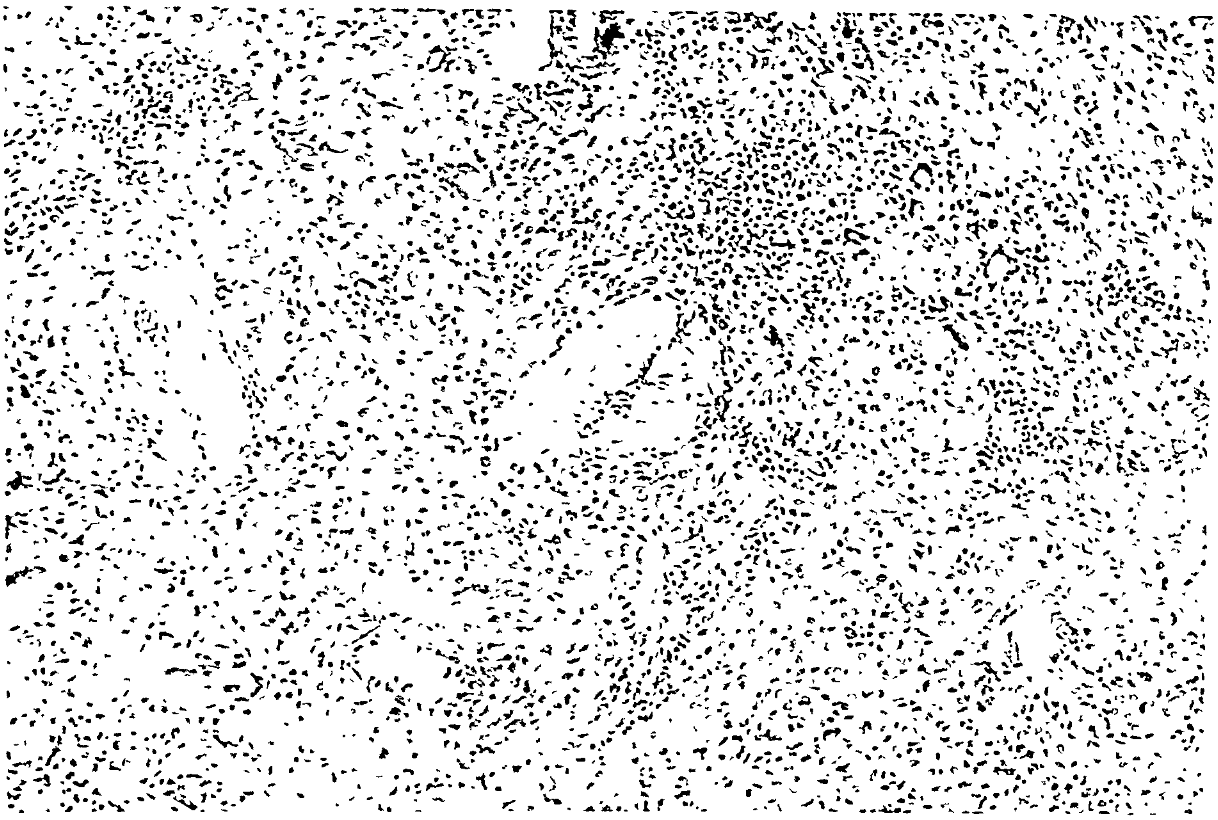

Fig. 3 (Case 4).—Low power photomicrograph (× 120), showing pronounced chronic granulomatous inflammatory reaction with foreign body giant cells. The tissue is almost totally composed of epitheloid cells with lymphocytes scattered in aggregates throughout.

Few children have been treated during the twenty years, and there were none in the 1946-1948 series. The treatment of 1 child, a boy aged 5 years, was satisfactorily completed, but he was a rare patient, being highly cooperative. Fewer treatments and smaller doses of the solution were used, along with a No. 22 bore short beveled needle.

### INDIVIDUAL STUDIES

CASE 1.—In 1947, a 32-year-old man who did much heavy lifting was given 24 injection treatments (14 cc. of Alparene No. 2 and 15 cc. of oil of sesame with 3 per cent phenol) for a left direct indirect inguinal hernia of two months' duration. On Sept. 29, 1950, he was operated upon at the Hartford (Connecticut) Hospital for a recurrence of the hernia. The operative report read "a solid mass was removed, the size of an apple; some small lumps were present [illegible] material in the center, which was due to the necrosis of the fibroplastic tissue." Frozen section "showed merely excessive fibrous or scar tissue reaction." Apparently, in this case, an excessive and concentrated amount of the asymmetric crystals were squeezed by the truss, muscular activity and tension producing a large amount of granulomatous tissue. The electrical impulses set up by the constant irritation of the concentrated and large amount of asymmetric crystals did not subside until a large mass had formed.

In 3 patients, who of their own volition had worn their trusses one to five years longer than the required time after completing their series of injections, large nongranular, nontender, solid masses developed in the treated groins. Evans and Zeit[12] stated, "Stress of some sort would hence be the functional stimulant where fibrosis is excessive."

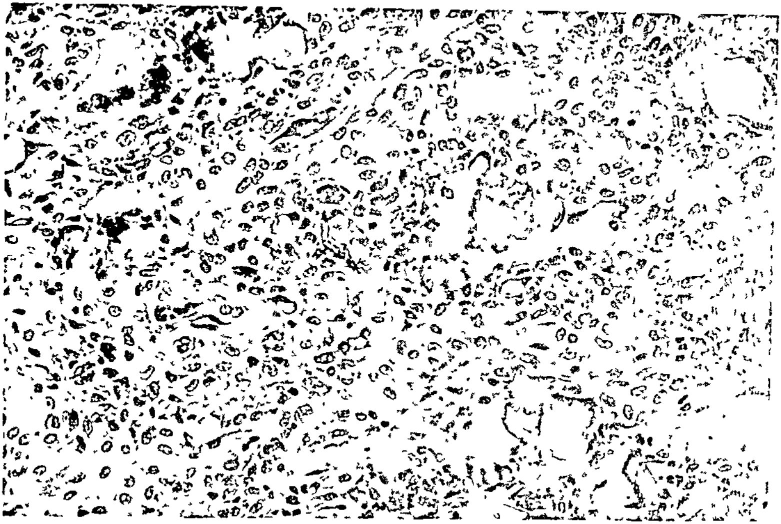

*Fig. 4 (Case 4).—High power photomicrograph (× 280) of specimen shown in Figure 3 Note the crystalline foreign bodies among the epitheloid cells and often adjacent to the giant cells*

CASE 2.—In 1945, a 50-year-old man was examined and a diagnosis of a right direct indirect inguinal hernia was made. This was verified by the insurance company examiner. The patient's hernia was treated by the ambulant method, and he was then reexamined by the same insurance doctor, who found no evidence of a right inguinal hernia at that time. He stipulated, however, that he would not approve payment by the insurance company until a considerable time had elapsed after this examination. Four years later, in 1949, he again examined the patient and found no recurrence, so he approved payment. Three years later the patient entered the Springfield Hospital for surgical observation. On April 25, 1951, he was operated upon by his surgeon for chronic recurrent appendicitis. The operative record read· "Internal inguinal ring was found to be obliterated. Peritoneum of hernial sac was also obliterated totally."

CASE 3.—In 1945, a 46-year-old man stated that he had undergone right herniotomies in 1942, 1943 and 1944, each with a recurrence. He had had the injection treatment for the right recurrent surgical hernia given by me in 1945. Nine years later, on Dec. 12, 1954, he was admitted to the Massachusetts Soldiers Home in Holyoke to be operated or for an irregular, hard lumpy mass in the right groin and a right recurrent hernia. On December 13, with the region under spinal anesthesia, he was operated upon. The operative record read: "A large, irregular mass was removed from the right groin. Hesselbach's triangle was firm. No hernia was found." The pathologic diagnosis was foreign body granuloma.

CASE 4.—On Jan. 3, 1956, a 55-year-old man was admitted to the Springfield Hospital for removal of a right hydrocele of eighteen months' duration, possibly due to trauma. He stated that he had undergone a left herniorraphy in 1936 and had had a right inguinal hernia treated by the injection method by me in 1946. Physical examination revealed no evidence of recurrent hernia in either groin, but the patient had a large right hydrocele that could be transilluminated. On Jan. 4, 1956, with spinal anesthesia, the right hydrocele was excised. With the prior knowledge and written consent of the patient, a biopsy specimen of the right groin was removed and was described as follows: "An incision was first made over Poupart's ligament and carried down to the aponeurosis of the external oblique. The entire granulomatous reaction was situated beneath the aponeurosis with the exception of the external ring where some reaction had occurred in the subcutaneous fat posterior to Scarpa's fascia. The external ring was entirely obliterated. The reaction could be palpated along the length of the inguinal canal to (approximately) the internal ring. The aponeurosis was incised along the length of the cord in this area, and a full thickness strip of aponeurosis down to the spermatic cord from internal to external ring was removed. The reaction was about ¼ inch thick at the region of the internal ring and about ½ inch thick at the external ring. It was noted that, for the most part, good planes of cleavage could be defined by sharp dissection over the aponeurosis and also over the spermatic cord. One could describe the granulomatous reaction as forming a tight glove about the spermatic cord. The pathologic diagnosis by Dr. William Kaufmann of this specimen, removed ten years after completion of the injection treatment, showed "foreign body granulomatous reaction containing a crystalline material" (Figs. 3 and 4).

CASE 5 (*Roentgen Data*).—In 1947, a 53-year-old man stated that he had had "lumps" in both groins for two years. During the same year, in my office, each of his direct indirect inguinal hernias was injected eighteen times, and the total amount used per groin was 26 cc. of Alparene No. 2 and 6 cc. of oil of sesame with 3 per cent phenol. Eight years later, on July 30, 1956, examination of both groins revealed no evidence of recurrences, and a moderate amount of granulomatous tissue was present in each groin. On the same day, roentgenologist Dr. John W. Turner took a survey film of the pelvic area, which showed "no difference in ossification of the pubic bones. There was no density observed in the inguinal areas to identify sites of the silicate injections."

*Sequelae and Complications.*—There have been no deaths among the 214 patients treated by injection in this series and no deaths with this procedure since I started treating inguinal hernias in this manner. Only 1 patient had an allergic reaction presumably due to an injection; "hives" developed but promptly disappeared after the administration of epinephrine. About 5 per cent of the 214 patients treated had a considerable amount of swelling and induration of the spermatic cord, which disappeared entirely within two to three weeks, aided by ice packs. Rarely was a patient sufficiently incapacitated to cause him to lose time from his daily routine.

There were no cases of local skin suppuration, inguinal abscesses or atrophied testes. Incidentally, during the first office visit, the testes are always examined along with the inguinal regions. On initial examinations, my 1946-1948 series revealed that about 4 per cent of the patients had atrophied testes on the same side as their hernias. Most of them stated that they had had mumps during childhood.

In the majority of cases, I have noted that post-injection recurrent hernias and their external rings were smaller than the original hernias.

Examination of this group of 214 patients revealed that 5 had small right hydroceles. Four had had direct indirect inguinal hernias and the other had a surgically recurrent hernia and stated that he had undergone two previous right herniotomies. Prior to this series and occasionally since 1948, a hydrocele did develop, usually following a deep injection. Unlike idiopathic and traumatic hydroceles, which often require surgical treatment, this post-injection type usually clears up permanently after one or two aspirations.

An excessive amount of nontender granulomatous tissue was produced in 23 patients with 26 inguinal hernias, and pronounced bulging was observed in the injected groins. In most cases it was undoubtedly due to the fact that some of the Alparene No. 2 had been injected into the subcutaneous fat anterior to the external oblique aponeurosis before the needle was withdrawn A nodular or granular feel was elicited in most of these groins This deposition of the chemical into the subcutaneous fat rarely occurs now, as I ascertain the exact position of the needle point by the subfascial fulcrum test before withdrawing it from the inguinal canal This has been done routinely for the past several years, and I make sure that the solution is injected entirely into the treated inguinal canal

Alparene No 2 has the faculty, when injected into subcutaneous fat, of forming small or large discreet nodules, which rarely become painful If the chemical injected *is highly concentrated or in too* large an amount, these nodules or granules, at pathologic examination, may present centers with necrosis, liquefaction and calcification

### COMMENT

"In communities in which the injection method has not been employed and may not even be known," Watson[13] advised, "the surgeon who contemplates the use of it, to discuss it with other doctors in his vicinity to familiarize them with it, so that if complications in the treatment should develop, he would not be charged with experimentation"

It is suggested that local surgeons *acquaint themselves with the granulomas* that form after the injection therapy Some of the patients in this series have been advised by other surgeons to have emergency operations for "incarcerated hernias" It is not known how many of the patients who were treated by injection and later by operation may only have had a granuloma rather than a recurrent hernia Such granulomas have also been mistaken for neoplasms and for tuberculosis

It is understandable that, if a patient previously treated by injection is operated *upon, the pathologist reports that a* hernial sac was observed The explanation of this, of course, is that the fibroplastic response against the injected material does not obliterate the sac, but either diminishes its width or holds it reduced within the internal ring Thus, the resulting granuloma acts either as a buttress against the internal ring or as a tight glove about the hernial sac, or both If now the granuloma is removed surgically, it stands to reason that a sac may be found and that repair is necessary As a matter of fact the sacs that have been examined have shown no significant reaction within the wall

Possibly the application of roentgen *rays, diathermy or ultrasonics to the* human groin into which piezoelectric quartz crystals have been injected may increase ionization in tissue and enhance the effect of local fibrosis This procedure might minimize the amount of material injected and still require very little roentgen dosage

In 1953, Zimmerman and Anson[11] stated "The final chapter on the sclerosing therapy of hernias has not yet, of course, been written"

### SUMMARY AND CONCLUSION

A series of 205 patients with 249 inguinal hernias treated by the ambulant injection method with Alparene No 2 is presented

The indications and prerequisites for successful therapy with this method are discussed in detail, and emphasis is laid upon the proper selection of cases for this type of therapy

With this treatment there were 34 recurrences or 13 7 per cent within two years and 27 recurrences, or 10 8 per cent after intervals ranging from two to ten years The end results at the end of eight to ten years showed 61 recurrences, or 24 5 per cent

No evidence of the development of malignant charge on the basis of the ambulatory injection treatment has been encountered over the ten-year period of observation.

The conclusion is reached that there is a place for the ambulant injection method of treating inguinal hernia in the male.

### SUMARIO E CONCLUSOES

Uma serie de 205 pacientes com 249 hernias inguinais foi tratada em ambulatorio com injeções de Alparene nº 2.

Discute as indicações e requisitos para o sucesso terapêutico e salienta a correta seleção dos casos para este tipo de tratamento. Nesta serie houve 35 recidivas ou 13.7% dentro de 2 anos e 27 recidivas ou 10.8% depois de 2 a 10 anos.

Os resultados finais ao fim de 8-10 anos mostraram 61 recidivas ou sejam 24.5%.

Não houve transformações malignas na observação feita durante 10 anos. Chega a conclusão de que há um lugar na terapêutica das injeções como método ambulatorio de tratamento das hernias nos homens.

### ZUSAMMENFASSUNG UND SCHLUSSFOLGERUNGEN

Eine Serie von 205 Kranken mit 249 Leistenbrüchen wurde mit ambulanter Einspritzung des Präparates Alparele No. 2 behandelt.

Die Indikationen und die Voraussetzungen für eine erfolgreiche Behandlung mit dieser Methode werden im einzelnen erörtert. Auf die sorgfältige Auswahl der Fälle für diese Art der Behandlung wird besonderer Wert gelegt.

Bei dieser Behandlung kam es innerhalb von zwei Jahren zu 34 Rückfällen (13,7%) und nach zwei bis zehn Jahren zu 27 Rückfällen (10.8%).

Die Endergebnisse nach acht bis zehn Jahren zeigten 61 Rückfälle oder 24,5%.

Während der zehnjährigen Beobachtungszeit stellten sich keine Entwicklungen bösartiger Veränderungen auf Grund der ambulanten Injektionsbehandlung heraus.

Der Verfasser kommt zu dem Schluss, dass die ambulante Injektionsmethode in der Therapie der Leistenbrüche des Mannes ihren Platz hat.

### RÉSUMÉ ET CONCLUSIONS

L'auteur présente une statistique de 205 malades atteints de 249 hernies inguinales, traités ambulatoirement par des injections d'Alparène No. 2.

Il décrit les indications et les conditions de succès de cette thérapeutique, qui exigent une bonne sélection des cas.

Il s'est produit 34 récidives (13.7%) au bout de deux ans, 17 récidives (10.8%) durant une période de deux à 10 ans, et 61 récidives (24.5%) après huit à dix ans.

Pendant un temps d'observation de dix ans il n'a été constaté aucune modification maligne due à cette thérapeutique. L'auteur en conclut qu'elle a sa place dans le traitement de la hernie inguinale chez l'homme.

### RIASSUNTO E CONCLUSIONI

Viene presentata una serie di 205 casi di ernia inguinale curata con il metodo delle iniezioni ambulatorie di Alparene.

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Author's Note: I wish to thank the following doctors for their permission to use cases reported in this paper: Case 1, Dr. Lewis A. Giffen; Case 2, Dr. Carl M. Binnig, and Cases 3 and 4, Dr. Lester Frank.

I also wish to acknowledge the invaluable assistance of Velma L. Bosworth, R.N., and Cecelia M. Curylo, R.N., in obtaining the case data presented and the cooperation of Mr. Ronauld L. Trembly of the Vernon Surgical Shop, Springfield, Massachusetts, in the development and construction of the trusses described.

Vengono descritte in dettaglio le indicazioni e le carateristiche del metodo, sopratutto nei confronti della scelta dei malati

Vi furono 34 recidive, pari al 13,7% entro i primi due anni e 27 (10,8%) fra il secondo e il decimo anno In complesso vi furono 61 recidive (24,5%) al termine di un periodo di osservazione di 8-10 anni Non vi furono casi di trasformazione maligna

Nel maschio, pertanto, questo metodo puo essere utilmente impiegato

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As long as the condition of the patient is such that the physician can cure him the physican is obliged to do all that is necessary to improve and restore his patient's health If, however, the physician decides on surgery as the best method of treatment, he is not authorized to proceed unless he has first secured his patient's consent By operating he cannot avoid injuring the patient's body and will often cause permanent physical changes If the patient is a minor, or mentally infirm, or insane, the physician's right to operate on the patient depends upon the consent of his legal representative

When a patient's life is endangered by a disease that is progressive unless brought under control, to tell the patient the truth might deprive him of the spiritual energy necessary for recovery Should such a reaction be an established medical experience the physician is obliged *not* to inform the patient of the nature of the disease

*—Honig*

# Genitourinary Surgery

# Tumors of the Renal Pelvis and Ureter

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THIS paper is based upon a study of 35 cases of carcinoma of the renal pelvis and ureter, 19 patients with tumors of the pelvis and 16 patients with tumors of the ureter. All of these patients were seen during the past twenty years, from 1936 through 1956, at Jefferson Hospital. Although we no longer consider either carcinoma of the ureter or carcinoma of the renal pelvis a rarity, they are relatively infrequent, and it therefore seems worth while to record our observations, particularly as regards the relation of the histologic type of the tumor to prognosis. It is not the purpose of this paper to attempt an exhaustive review of the literature or to discuss in detail the causation of these tumors, although a brief review of the observations up to the time of writing, as compared to our own observations, will be made.

*Incidence*.—Tumors of the renal pelvis and ureter are considered uncommon, although the incidence has increased decidedly during the past twenty-five years. This is undoubtedly due to increased awareness of these tumors and improved diagnostic methods. In 1955, Whitlock, McDonald and Cook stated that 312 cases of carcinoma of the ureter had been reported in the literature; since that time a number of cases have been added. Scott, in his review of tumors of the renal pelvis in Campbell's *Urology*, in comparing pelvic tumors to parenchymal tumors re-

TABLE 1.—*Age of Patient*

| *Age* | *Number* | *Pelvis* | *Ureter* |
|---|---|---|---|
| 20-29 | 2 | 1 | 1 |
| 30-39 | 4 | 1 | 3 |
| 40-49 | 5 | 5 | 0 |
| 50-59 | 7 | 3 | 4 |
| 60-69 | 7 | 4 | 3 |
| 70-79 | 3 | 1 | 2 |
| 80-90 | 1 | 1 | 0 |
| Unknown | 6 | 3 | 3 |
| Total | 35 | 19 | 16 |

TABLE 2.—*Site of Growth (Ureteral Tumor)*

| *Portion of Ureter* | |
|---|---|
| Upper third | 1 |
| Middle third | 0 |
| Lower third | 9 |
| Lower and middle thirds | 3 |
| Upper and lower thirds | 1 |
| All portions | 1 |
| Unknown (autopsy) | 1 |
| Total | 16 |

TABLE 3.—*Site Involved*

| | *Ureteral* | *Pelvic* |
|---|---|---|
| Right | 8 | 10 |
| Left | 6 | 9 |
| Unknown | 2 | 4 |
| Total | 16 | 19 |

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From the Department of Urology, Jefferson Medical College and Hospital, Philadelphia.
Read at the Twenty-[illegible] Annual Congress of the United States and Canadian Section, International College of Surgeons, Chicago, [illegible]
[illegible]

corded the incidence as 1:14 respectively.

*Age:* Both tumors of the pelvis and tumors of the ureter are most common in the fifth and sixth decades of life. There is a tendency for benign and low-grade tumors of the ureter to occur in younger persons. Table 1 illustrates the age distribution in our own series.

*Sex:* Both types of tumor are more common in the male than in the female, with a proportion of approximately 3:1. In this series there were 15 male and 4 female patients with tumors of the renal pelvis, 12 male and 4 female patients had tumors of the ureter.

*Site of Growth:* Study of the cases previously reported reveals that most (two-thirds or more) of the ureteral tumors occur in the lower third of the ureter. A significant percentage of both types of tumore are multiple, probably about 25 per cent. In this series, 13 of 16 ureteral tumors involved the lower third, 4 of these involving both the middle and lower thirds (Table 2).

Tables 11 and 12 illustrate the multiplicity of tumors in this series, 7 of 19 tumors of the renal pelvis and 11 of 16 ureteral tumors being multiple.

Some reports have stated that the right side is involved somewhat more often than the left. This was not, however, the experience of Whitlock, McDonald and Cook, who found, in a review of 33 patients with carcinoma of the ureter, that the left side was involved 21 times and the right side 12 times. Table 3 illustrates the side involved in this series, there being no significant difference.

*Symptoms.*—The majority of patients complain of hematuria, this having previously been found to be present in about 70 per cent of the cases. A lesser percentage (about 60 per cent) in a recent series by Cook, Culp, McDonald and Ut complained of lumbar or abdominal pain Dysuria and frequency of urination are

TABLE 4.—*Presenting Symptom—Carcinoma of Renal Pelvis and Ureter*

| | *Hematuria* | | *Lumbar Pain* | | *Abdominal Pain* | | *Frequency* | | *Dysuria* | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Duration* | *Ureter* | *Pelvis* | *Ureter* | *Pelvis* | *Ureter* | *Pelvis* | *Ureter* | *Pelvis* | *Ureter* | *Pelvis* |
| Less than 1 week | 3 | 4 | 1 | | | 2 | | | | |
| Less than 1 month | 1 | 2 | 1 | | | | | | 1 | |
| 1-3 months | 2 | 5 | | 3 | | | | | | |
| 3-6 months | 1 | 1 | 2 | | | 1 | | | | |
| 6-12 months | 1 | 1 | | | | | | 1 | 1 | 1 |
| 1-2 years | | | | 1 | | | | | | |
| 2-3 years | | | 1 | | | | | | | |
| 3-4 years | | | | | | | | | | |
| 4-5 years | 1 | | | | | | | | | |
| 6-7 years | | | 1 | | | | | | | |
| 16 years | 1 | | | | | | | | | |
| 31 years | | | 1 | | | | | | | |
| Totals | 10 | 13 | 7 | 4 | 0 | 3 | 0 | 1 | 2 | 1 |

Symptoms Unknown:
Carcinoma of the pelvis—4
Carcinoma of the ureter—3

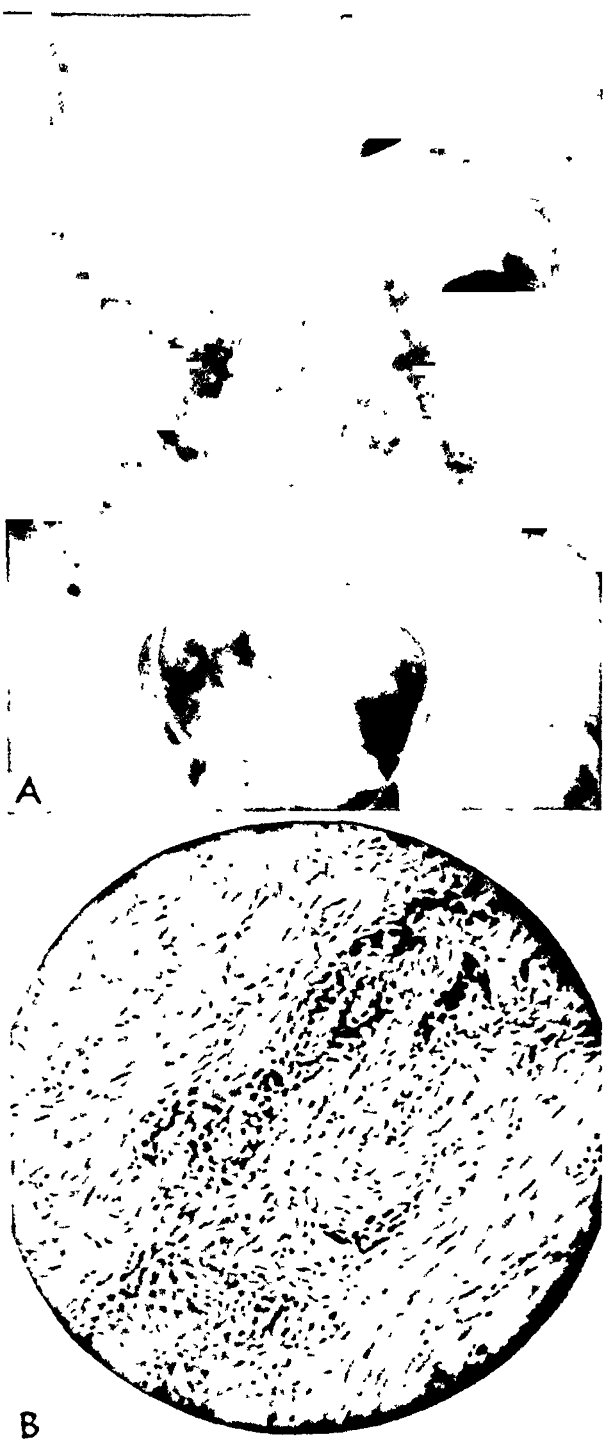

Fig. 1.—*A*, retrograde pyelogram showing filling defect in lower and middle calyceal systems. *B*, histologic section showing poorly differentiated squamous cell carcinoma of the renal pelvis (× 200), in a [illegible]-year-old white man who died fifteen months after nephrectomy, of metastasis; the patient also had a squamous cell carcinoma of the bronchus, for which pneumonectomy was done. It could not be determined whether two primary carcinomas existed, or whether one was metastatic.

occasional complaints. An abdominal mass is occasionally noted by the patient. In this series, 10 of 16 patients with carcinoma of the ureter had a history of hematuria (62.5 per cent), and 13 of 19 patients with carcinoma of the pelvis had hematuria (67.7 per cent). Of the patients with ureteral tumors, 7 of 16 (43.75 per cent) complained of lumbar pain. Table 4 presents the clinical symptoms and their duration in this series. It is interesting to note that 1 patient had hematuria intermittently for four or five years and another for sixteen years. It seems likely that each of these 2 patients may have had a benign, slow-growing, tumor that later underwent malignant change.

*Diagnosis.* — *Cystoscopic*: The cystoscopic data should often lead one to suspect the diagnosis. In some patients with carcinoma of the ureter the tumor may be seen to protrude from the ureteral orifice. This occurred in 6 of the 16 cases of ureteral carcinoma in this series. The presence of a vesical tumor, especially one situated near the ureteral orifice or on the base of the bladder, should make one think of the possibility of tumor in the upper part of the tract. Edema and congestion, occasionally associated with dilatation, sometimes involve the affected ureteral orifice. The excretion of phenolsulphthalein or indigo carmine is often decreased or absent on the affected side. The presence of these signs in this series is indicated in Table 6, 12 of the 16 ureteral tumors and 7 of the 19 pelvic tumors having been associated with significant cystoscopic observations.

Two patients with primary carcinoma of the renal pelvis and secondary involvement of the lower part of the ureter also had tumor protruding from the ureteral orifice. The egress of bloody urine from the ureteral orifice on the involved side is often observed, although it was only noted 7 times in this series of 35 cases.

TABLE 5.—*Roentgen Data*

| | *Carcinoma of Ureter* | *Carcinoma of Renal Pelvis* |
|---|---|---|
| Filling defect with or without deformity | 6 | 12 |
| Hydronephrosis and hydroureter | 3 | |
| Hydronephrosis | 2 | 2 |
| Hydroureter | 1 | |
| Non-function of kidney on intravenous urogram | 3 | 2 |
| Calculi | | 2 |
| Normal roentgen picture | 3 | |
| Unknown | 3 | 5 |
| Totals | 21 | 23 |

TABLE 6.—*Cystoscopic Data*

| | *Bloody Urine from Orifice* | *Tumor Protruding from Orifice* | *Bladder Tumor* | *Dilated Orifice* |
|---|---|---|---|---|
| Ureter | 4 | 6 | 1 | 1 |
| Pelvis | 3 | 2 | 1 | 1 |
| Totals | 7 | 8 | 2 | 2 |

TABLE 7.—*Carcinoma of Ureter: Incidence of Types and Grades*

| *Grade* | *Papillary* | *Papillary and Infiltrating* | *Infiltrating* |
|---|---|---|---|
| 1 | 5 | 0 | 0 |
| 2 | 1 | 4 | 0 |
| 3 | 0 | 3 | 2 |
| 4 | 0 | 0 | 1 |
| Totals | 6 | 7 | 3 |

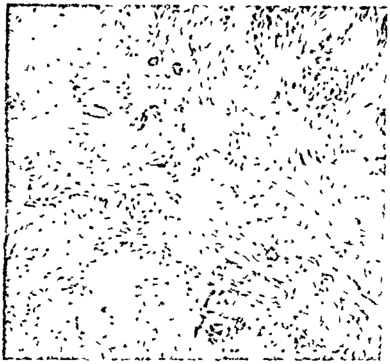

FIG. 2.—Histologic section showing squamous cell carcinoma of renal pelvis with infiltration into parenchyma. × 67.

Scott, in *Campbell's Urology,* stated that tumor of the bladder is present in 20 to 25 per cent of the patients with carcinoma of the renal pelvis; similarly, it is present in a significant number of patients with ureteral carcinoma. In 1 of the cases in this series the diagnosis of carcinoma of the ureter was not made for two years after the patient was treated for carcinoma of the bladder, despite the initial presence of mild hydroureter on the in travenous urogram. This patient died c metastasis three years and eight mont' after a diagnosis of vesical carcino a was made; the ureteral tumor was di nosed as papillary carcinoma, Grade I without infiltration, whereas the vesical carcinoma was a papillary carcinoma Grade III, with infiltration.

Catheterization of the involved ureter often reveals complete obstruction in the tumor area. In some instances the catheter may be passed but its passage is followed by bleeding from the ureteral orifice around the catheter, or bleeding through the catheter, the latter usually disappearing when the tumor area is passed.

*Roentgen Studies:* Excretory urographic studies reveal significant signs in the majority of cases. Hydronephrosis and hydroureter are common. A filling defect in the involved area, often of the "goblet-shaped" variety, is frequently seen, or the involved area may appear as a point of stricture in the ureter. Decreased or absent function is common. The roentgenographic data on this series are

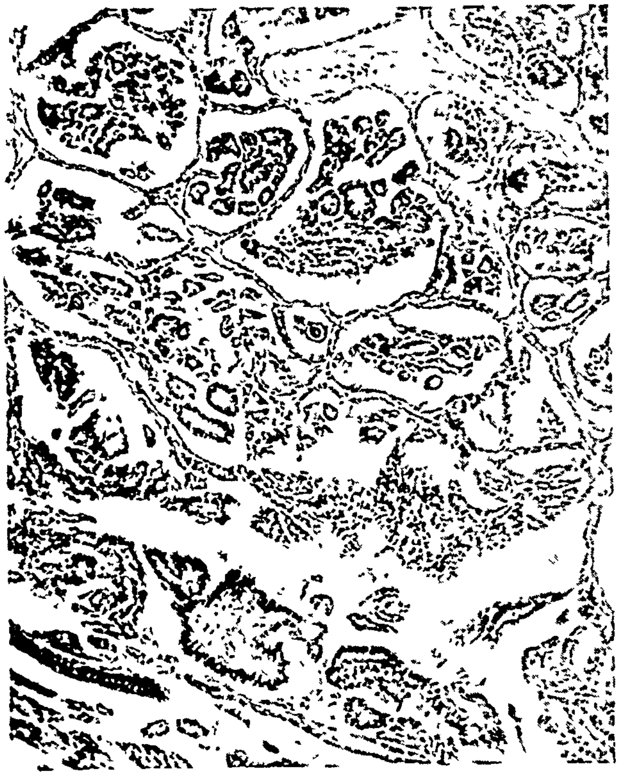

Fig. 3.—Histologic section showing papillary cystadenocarcinoma of renal pelvis. × 100. Patient was a 35-year-old white woman with a complaint of hematuria; retrograde pyelogram demonstrated filling defect in renal pelvis. Patient died of metastases thirty-nine months after operation. (*Courtesy of Dr. David M. Davis.*)

recorded in Table 5. It is significant that the roentgen signs are positive in the majority of cases.

*Etiologic and Pathogenic Background.*—It is not within the scope of this article to deal with an exhaustive review of the literature concerning the etiologic factors. Much has been said previously of the etiologic theories advanced. Maclean and Fowler, as well as many others have pointed out that proliferative or metaplastic changes may take place as a result of chronic irritative changes in the ureter or pelvis. Proliferative changes include ureteritis granulosa, pyelitis granulosa, ureteritis polyposa and pyelitis polyposa. If there is a downward proliferation of the epithelium, however, solid nests of cells may be seen, as originally described by von Brunn in 1893. This may result in ureteritis cystica or pyelitis cystica. Fagerstrom made a detailed study of ureters removed from 120 cadavers in order to study epithelial aberrancies and to determine the possible etiologic significance in cases of tumor of the ureteral mucosa. He concluded that the epithelial buds and crypts were bizarre expressions of epithelial hyperplasia and was unable to support the theory of "cell nest" formation proposed by von Brunn. Fagerstrom was unable to develop any evidence that solid epithelial buds are directly associated with the origin of solid tumors of the urinary epithelium. Maclean and Fowler pointed out that both squamous and mucinous metaplasia may take place, and presented patients illustrating these changes. They mentioned, as irritants that may produce proliferative or metaplastic changes, (1) long-continued bacterial infection, (2) obstructive uropathic conditions, (3) calculus, (4) avitaminosis, especially of vitamin A, (5) hormonal imbalance (6) toxic products of metabolism and (7) specific carcinogens. They reported the fourth case of mucinous adenocarcinoma of the renal pelvis. The fifth case of such a tumor is presented as a part of this series, as are 3 cases of squamous cell carcinoma of the renal pelvis. Etiologic factors that ex-

TABLE 8.—*Carcinoma of Renal Pelvis: Incidence of 4 Types and Grades*

| *Grade* | *Papillary* | *Papillary and Infiltrating* | *Infiltrating Carcinoma* | *Mucous Adenocarcinoma* | *Papillary Adenocarcinoma* |
|---|---|---|---|---|---|
| 1 | 1 | 0 | 0 | 0 | 0 |
| 2 | 4 | 3 | 0 | 0 | 0 |
| 3 | 0 | 3 | 4 | 1 | 1 |
| 4 | 0 | 0 | 2 | 0 | 0 |
| Totals | 5 | 6 | 6 | 1 | 1 |

plain the multiplicity of ureteral tumors are lymphatic permeation, surface implantation and multicentric origin. Most observers agree that a multicentric agent is responsible for the stimulation of the multiple tumors that may appear in the urinary tract. There seems to be little pathologic support for lymphatic permeation. Various investigators have been unable, for the most part, to transplant tumor cells in the experimental animal, which argues against surface implantation. On the other hand, surface implantation is supported by the fact that the tumors usually seem to follow the flow of urine and to be most common in the area of the ureter where temporary stasis is present. Bothe, in 1943, studied 6 tumors of the ureter and 4 of the renal pelvis, stated that he observed similar changes

TABLE 9.—*Operations Performed for Ureteral Tumor*

| | |
|---|---|
| Nephro-ureterectomy with cuff of bladder | |
| One-stage | 8 |
| Two-stage | 3 |
| Nephrostomy (metastatic carcinoma) | 1 |
| Cystoscopic removal and fulguration | 3 |
| No operation (inoperable) | 1 |
| Total | 16 |

TABLE 10.—*Operations Performed for Tumor of Renal Pelvis*

| | |
|---|---|
| Nephro-ureterectomy with cuff of bladder | |
| One-stage | 3 |
| Two-stage | 5 |
| Nephrectomy and partial ureterectomy | 8 |
| Unknown | 2 |
| No operation | 1 |
| Total | 19 |

TABLE 11.—*Carcinoma of the Renal Pelvis; Multiple Growths*

| | |
|---|---|
| Renal pelvis and lower third of the ureter | 3 |
| Renal pelvis and bladder | 2 |
| Renal pelvis and proximal third | 1 |
| Renal pelvis, proximal and distal thirds | 1 |
| Total | 7 |

TABLE 12.—*Carcinoma of the Ureter; Multiple Growths*

| | |
|---|---|
| Lower third and bladder | 5 |
| Lower and middle thirds | 3 |
| Upper and lower thirds | 1 |
| All portions | 1 |
| Unknown | 1 |
| Total | 11 |

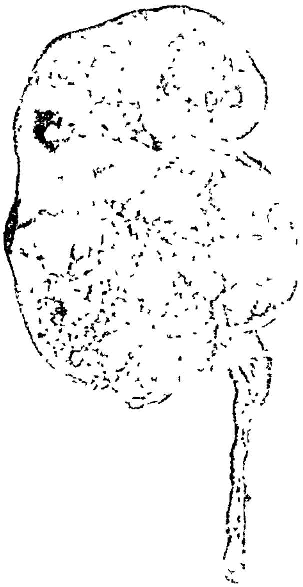

Fig. 4.—Gross specimen showing invasive papillary carcinoma of renal pelvis and upper part of ureter; lower part of ureter (not shown) was also involved. Patient was a 42-year-old white man who underwent nephroureterectomy with excision of a bladder cuff; he remained well for five and one-half years. Recently he had a recurrent papillary carcinoma of the bladder

mm. in width and 3 to 18 mm. in thickness. The average size of the left and the right suprarenal differed little; that of the left averaged 50 by 23 by 7 mm. and that of the right 50 by 27 by 6 mm. Topographically considered, the respective glands were located between the upper pole of the kidney and the abdominal portion of the aorta. The glands were enclosed in the renal fascia but were separated from the kidney by a pad of fat, and from the aorta not only by fat and connective tissue but also by the celiac ganglion. The right suprarenal gland was contiguous with the inferior vena cava, which crossed its ventral aspect. It was more closely confined by neighboring structures and at times extended over the superior pole of the kidney. Since the left suprarenal was invariably situated at the upper medial aspect of the kidney, a position often taken likewise by the right suprarenal, the glands can just as appropriately be called adrenal glands, as they often are.

The concept gained from many texts on anatomy that three unbranched, i.e., stem arteries supply each gland is obsolete and false. In Table 2 the arteries are classified according to the region of the gland they supply, viz., the superior, middle and inferior portions. The superior suprarenal arteries supply the superomedial, the middle suprarenal, the anteromedial aspect; the inferior suprarenal, the posterior and inferolateral aspect. Sites of origin of the superior suprarenals are grouped into those that arise from: (1) the inferior phrenic before its division into an anterior and posterior branch, (2) its posterior branch and (3) both sources.

The superior suprarenal arteries, as shown in Table 2, arose almost exclusively from the inferior phrenic. In 1 case the supply was directly from the aorta. In 4 cases the superior area of the left suprarenal gland received some blood directly from the aorta in addition to the supply coming from the inferior phrenic.

The middle suprarenal (usually single) had varied sites of origin, these being, on one side or the other as follows: the inferior phrenic (24 cases), the aorta (18 cases), the renal artery (4 cases) and the celiac axis (3 cases). Often the middle suprarenal was replaced, on one side or the other, by the superior (9 cases) or the inferior (41 cases) suprarenal artery. In 1 specimen with a middle suprarenal, the specific area was partially supplied by the inferior suprarenal.

The inferior suprarenal arteries are the most important, since they supply the thickest and largest part of the gland. They may be grouped into proximal, mid-

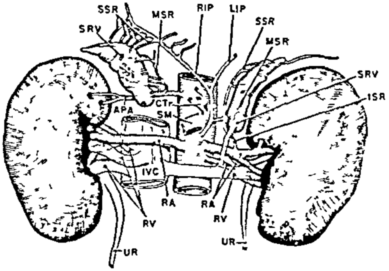

Fig. 5—N ♂, 37 years. Sample of bilateral dual renal arteries. On the left, both are hilar; on the right, one is hilar with a precaval course, the other is a superior polar that bifurcates before entry. In the renal segmentation terms of Graves applied here, branches to the right kidney, purportedly, comprise the apical (2), upper, middle, posterior and lower segmental branch In the left kidney, the upper renal, after giving off the posterior segmental gives rise to the apical, upper and middle segmental, while the lower artery becomes the lower segmental. Sample shows that an aortic superior polar may be considerably more than a renal artery, for here, it (*APA*) furnishes the main blood supply to the medial and inferior surfaces of the suprarenal gland (*MSR*). Inferior phrenics (*RIP*, *LIP*) arise via a common trunk from aorta below superior mesenteric and suppl[illegible] (*SSR*). On the left, the i[illegible]'s origin from [illegible] (*MSR*) and anastomoses with the inferior phrenic

the inferior vena cava as far as the renal veins is stopped, or possibly even reversed, while the onward flow beyond them is rapid.

It may also be noted that at high intra-abdominal pressures, in spite of the steep fall in the gradient beyond the renal veins, there is still a kink at the level of the diaphragm and an even sharper fall beyond this point. On the basis of my previous interpretation this would suggest that there is no stoppage of hepatic (and portal) flow at high intra-abdominal pressure levels.

*Radiographic observations.* These experiments were performed jointly with Dr C. J. Hodson, Assistant Director of the Radiological Department, University College Hospital, who was responsible for the radiographic interpretations.

In 4 rabbits a catheter was inserted into the left femoral vein so that its tip almost reached the junction of the iliac veins. A small quantity ($\frac{1}{4}$-$\frac{1}{2}$ c.c.) of a contrast medium—a super-saturated solution of "Diodone"—was injected through the catheter and was seen on the screen as an opaque blob which entered the blood stream and moved with it up the inferior vena cava. The volume of fluid injected was so small that it could not have influenced the volume, velocity or direction of the stream in which it moved.

At normal intra-abdominal pressures, the blob of Diodone moved quickly and steadily up the inferior vena cava to the heart, where it disappeared by mixing and dilution with blood.

When the intra-abdominal pressure was raised to about 30 cm. of water by inflating the peritoneum, the opaque blob was seen to move slowly up the abdominal vena cava as far as the renal veins, and there to join a more swiftly moving stream of blood. Before reaching the renal veins its progress was slower than it had been at normal intra-abdominal pressure; beyond them, movement was at least as rapid.

When intra-abdominal pressure was raised to 40 cm. of water, the opaque medium no longer moved up the inferior vena cava but passed down the iliac vein of the opposite (right) side. With the intra-abdominal pressure still at 40 cm. of water, the catheter was pushed up the inferior vena cava until its tip lay opposite the left (lower) renal vein. From this point Diodone passed rapidly up the vena cava to the heart as before and without any delay. The critical pressures which caused deviation of the stream from the abdominal vena cava were 40 cm. of water in the first two rabbits and 45 cm. of water in the third; the fourth rabbit died before the exact pressure had been determined. But it is probable that the diversion was a gradual process.

By injecting relatively large quantities of Diodone (about 1 c.c.) the path beyond the iliac vein could be traced. The stream was seen to enter the ascending lumbar vein, which passes upwards on either side in front of the transverse processes of the lumbar vertebræ, deeply embedded in muscle which protects it to some extent from compression when the abdomen is distended. At the level of the 12th thoracic vertebra it joins the azygos vein, and so the diverted blood enters the superior vena cava. When large quantities of contrast medium are injected, some of it enters the inferior vena cava under the force of injection. In these circumstances the stream escapes from the vena cava into the ascending lumbar veins through lumbar veins which communicate transversely between the two main vessels. They also connect the ascending lumbar route with the paravertebral plexus of veins, and it is probable that some blood ascends in this plexus. This collateral circuit was detected in all four rabbits when the intra-abdominal pressure was high. Altering the posture of the animal had no effect on the path taken.

It is possible that at very high intra-abdominal pressures, some of the renal blood may find its way into the ascending lumbar veins via a lumbar vein which joins the vena cava very close to the point of entry of the left renal vein.

The diversion of blood away from the lower end of the inferior vena cava at high intra-abdominal pressures explains why the vena-caval pressure does not rise as high as the portal pressure.

### *Experiments on blood distribution*

Conclusions from these experiments relate to blood pressure and velocity of flow. To complete the investigations the abdominal blood volume was measured before, during and after distension of the abdomen. For this purpose Welcker's (1858) method was chosen as it allows accurate separation of abdominal blood from the rest.

Fifteen anæsthetised heparinised rabbits were divided into 3 groups. Those in the first group, serving as controls, were not subjected to experiment, but after 30 minutes they were killed by the intravenous injection of a Nembutal and chloroform mixture which produced almost simultaneous arrest of heart and respiration. In the second group intra-abdominal pressure was raised 20-50 cm. of water for 30 minutes by inflation of the peritoneum or sometimes of the colon, and the animals were killed before pressure was released. Those in the third group had the same treatment, but were killed 2-10 minutes after pressure had been released.

As soon as an animal was dead, the inferior vena cava was clamped above the diaphragm and ligated below the left (lower) renal vein. In this way movement of blood into or out of the abdominal veins during the subsequent manipulations was prevented. Five cannulas were then inserted as shown in fig. 5. They were already connected to rubber tubing, which had been clamped. All clamps were removed and warm saline was injected through cannulas 1 and 2; blood and perfusion fluid was collected from cannulas 3, 4 and 5 into flasks containing saponin to produce hæmolysis.

It will be seen from the diagram that saline from cannula 1, being

to Howarth and Sherlock (personal communication), is unaffected by the abdominal distension of ascites; and (3) the velocity of blood-flow in the aorta, which is very unlikely to be increased by a rise of pressure in the abdomen. It follows that the work done by the heart is not significantly increased by abdominal distension.

It is probable that the embarrassment to the circulation is an indirect one, resulting from interference with respiration by the raised pressure in the abdomen. This hypothesis is supported by the obvious dyspnœa, the striking impairment of tolerance to abdominal distension when fluid is present in the pleura, and the evidence of cardiac ischæmia in the electrocardiograms. Further experimental evidence in support of this view was put forward by Gatch and Battersby (1942*b*).

### *The venous pressure in abdominal distension*

The blood pressure in a vessel is expressed by a derivation of Laplace's formula (Thompson, 1942) :—

$$P = p + \frac{T}{R}.$$

Where P is the fluid pressure in the vessel, T the tension in the wall, R the radius of the vessel and $p$ is the external pressure.

When there is no external pressure on a vessel, the blood pressure varies with the tension in the wall, which is due to the action of the heart. But if the external pressure rises the blood pressure rises by equal increments. This explains the behaviour of the venous pressure in the abdomen. The formula applies only to vessels which are filled, but in so far as the venous pressure exceeds the intra-abdominal pressure, it is reasonable to assume that the veins are filled.

It follows that since the rise of pressure inside and outside the abdominal veins is equal, there is no tendency for them to collapse. A different situation arises, however, if instead of a general rise of intra-abdominal pressure, local pressure is applied to a vessel. In this case there will be no rise of pressure in the vein except that due to the damming back of blood, and the vessel is more likely to be obstructed. It might be expected, therefore, that in pregnancy the vena cava would be collapsed by pressure on it from the enlarged uterus, and from the weight of the fœtus when the mother is lying supine; blood would presumably be diverted up the ascending lumbar vessels.

### *The maintenance of the abdominal circulation*

There is evidence that blood flow in the abdomen is not greatly reduced by a rise of pressure.

(1) My experiments suggest that while the volume of blood in the abdominal vessels is not reduced by distension, the velocity of flow

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discrepancies in this group are due to an experimental error which does not affect the others. This could be explained by assuming that in animals killed while the abdomen is distended a variable quantity of blood is driven from the abdomen after death by the high intra-abdominal pressure, while in the first and last groups there are no post-mortem pressure differences between abdomen and thorax and no movement of blood takes place. This seems the most probable explanation. The inference is that in the group with raised intra-abdominal pressure the high results—abdominal blood volume 57 per cent. of the total—are the least distorted; and these are higher than any of the results in control animals.

TABLE

*Abdominal blood volumes expressed as a percentage of the total blood volume*

| Control animals | During raised intra-abdominal pressure | After decompression |
|---|---|---|
| 51 | 57 | 43 |
| 51 | 57 | 43 |
| 49 | 52 | 46 |
| 49 | 43 | 45 |
| 52 | 47 | 45 |
| Mean 50·4 per cent. | 51·2 per cent. | 44·4 per cent. |

The table also shows that the ratio of abdominal to total blood volume is smaller after decompression of the abdomen than in control animals which had not been subject to raised intra-abdominal pressure, by about a tenth (44·4-50·4 per cent.). This difference is highly significant in spite of the small numbers in each group ($t = 7{\cdot}0$; $P = 0{\cdot}001$).

It must be concluded, therefore, that the relative abdominal blood volume falls immediately after decompression, and that this fall lasts 10 minutes or more. There is also a suggestion that while intra-abdominal pressure is raised the relative abdominal blood volume is higher than normal. In other words the splanchnic pool may tend to fill as a result of abdominal distension, and it empties to some extent after decompression, the opposite of what has been generally expected. But the volume changes are small.

## DISCUSSION

### *Circulatory embarrassment from abdominal distension*

The work done by the heart is based on (1) the arterial blood pressure which, as shown in these experiments and those of other workers, does not alter appreciably in response to a moderate rise of intra-abdominal pressure; (2) the cardiac output, which, according

to Howarth and Sherlock (personal communication), is unaffected by the abdominal distension of ascites; and (3) the velocity of blood-flow in the aorta, which is very unlikely to be increased by a rise of pressure in the abdomen. It follows that the work done by the heart is not significantly increased by abdominal distension.

It is probable that the embarrassment to the circulation is an indirect one, resulting from interference with respiration by the raised pressure in the abdomen. This hypothesis is supported by the obvious dyspnœa, the striking impairment of tolerance to abdominal distension when fluid is present in the pleura, and the evidence of cardiac ischæmia in the electrocardiograms. Further experimental evidence in support of this view was put forward by Gatch and Battersby (1942*b*).

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### *The maintenance of the abdominal circulation*

There is evidence that blood flow in the abdomen is not greatly reduced by a rise of pressure.

(1) My experiments suggest that while the volume of blood in the abdominal vessels is not reduced by distension, the velocity of flow

in the veins is at least as great as at normal pressure, except in so far as blood is diverted up the ascending lumbar route. These facts were established for intra-abdominal pressures up to about 40 cm. of water.

(2) Gatch *et al.* (1927) measured blood flow through a loop of small intestine in dogs while it was being inflated. They concluded that blood flow was inversely proportional to pressure, but their results show that when intra-visceral pressure was raised to 30 cm. of water, blood flow was reduced only from 18 to 17 c.c. per minute, and in some of their animals it rose.

Yet the arterio-venous pressure difference is appreciably reduced by abdominal distension, since the rise in venous pressure is not balanced by a corresponding rise of arterial pressure. These facts can only be reconciled by postulating a reduction in resistance to flow, for from Poiseuille's formula $V \propto Pr^4$ ($V$ = volume, $P$ = pressure difference and $r$ = radius of vessel).

The main resistance to flow is in the arterioles. It is evident that if an arterial pressure of 100 mm. of mercury in the normal rabbit is required to support a capillary pressure of 10 cm. of water, an arterial pressure of 110 mm. of mercury will be inadequate to support a capillary pressure of say 45 cm. of water, which would be required to maintain flow under abdominal distension, unless there were a reduction of the arteriolar resistance.

I conclude that a rise of intra-abdominal pressure is associated with arteriolar dilatation in the abdominal viscera and that maintenance of their circulation is dependent on this reaction.

There is no reason to suppose that capillaries, any more than veins, need be collapsed or dilated as a result of raised intra-abdominal pressure, but the question may be asked whether any reduction of capillary resistance is needed to facilitate blood flow. Evidence is accumulating that the abdominal viscera are supplied with relatively direct pathways across the capillary beds, such as those in the kidney demonstrated by Trueta *et al.* (1947), and in the stomach by Barclay and Bentley (1949). Prinzmetal *et al.* (1948) showed that glass spheres much larger than ordinary capillaries could be passed through the circulation of the liver, spleen and lungs of a variety of live animals. Whether these pathways are in fact opened in abdominal distension is a matter for speculation. But if it be accepted that blood flow is not much reduced by a moderate rise of intra-abdominal pressure, the findings of reduced hepatic function by Bradley (1946) and of a 25 per cent. reduction in renal function by Bradley and Bradley (1947) when intra-abdominal pressure was raised to 20 mm. of mercury may be taken as an indication that blood is diverted from the capillaries.

The marked fall in blood pressure which I found in some animals when intra-abdominal pressure was raised after sympathectomy was probably due to a failure to maintain the circulation through the

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The marked fall in blood pressure which I found in some animals when intra-abdominal pressure was raised after sympathectomy was probably due to a failure to maintain the circulation through the abdomen because of an inability to adapt the small vessels for flow under high pressure.

A possible explanation for the abdominal blood-volume variations is provided by the hypothesis of arteriolar dilatation and constriction. Krogh (1921) noticed that when an arteriole was dilated its capillaries took their blood from the axial stream, rich in red cells, and that when it was constricted its capillaries became almost drained of cells as they skimmed off only the marginal stream, predominantly plasma. These changes could account for the blood-volume variations as estimated by Welcker's method. If this were the explanation it would follow that there is probably no alteration in the true abdominal blood volume as a result of pressure changes.

### *Collapse following abdominal decompression*

The danger of collapse following paracentesis of the abdomen or decompression of the distended bladder is well known, yet I have failed to find any adequate account of this complication and Wangensteen and Scott (1928) had the same lack of success. Brams and Golden (1933-34), however, refer to two cases of temporary circulatory collapse after withdrawal of ascitic fluid from 10 patients and in one the patient had collapsed on two occasions; they imply that this sequel is not unusual. It seems likely that while collapse after decompression is not uncommon it is rarely irreversible, or troublesome enough to warrant publication of reports.

Both the pairs of workers just mentioned produce evidence that faintness and collapse are associated with a fall of arterial blood pressure, though I was unable to produce a fall below the initial level in my rabbit experiments by sudden abdominal decompression. If my hypothesis that abdominal distension is accompanied by arteriolar dilatation is accepted, an occasional large fall in blood pressure after decompression could be explained by postulating a failure of the compensatory vasoconstriction.

Although the general intra-abdominal pressure in late pregnancy is not known, the pressure fall as a result of labour might be compared with the decompression of a severely distended bladder, both in degree and in its position in the abdomen. The gross discrepancy between the incidence of irreversible shock following labour and after decompression of the bladder suggests that the fall of intra-abdominal pressure is not, by itself, an important feature of obstetric shock.

### *Application of experimental results to man*

The experiments reported above were performed on anæsthetised rabbits lying on their backs, and the same conditions apply to most of the previous work done in this field. Results so far obtained on man, however, are very like those found in rabbits and dogs as regards arterial and venous blood pressure. The anatomy of the abdominal veins and of the ascending lumbar system is essentially the same in rabbits and in man, and it is known that the latter pathway is taken when the inferior vena cava is ligated at operation (Thebaut and Ward, 1947). Man, too, is forced to revert to the horizontal when abdominal distension becomes severe, and it is in this position that decompression is carried out; so that the conclusion which emerges from studies on rabbits may well be applicable to man.

## SUMMARY

The following conclusions are drawn from the acute experiments on rabbits described above.

1. Evidence that circulatory embarrassment from abdominal distension is due to interference with respiration is supported by the calculation that the work done by the heart is not increased.
2. The veins of the portal and vena-caval systems do not easily collapse in abdominal distension, since the pressure in them is higher than that in the abdominal cavity.
3. When the intra-abdominal pressure exceeds 40 cm. of water, the renal and probably the portal blood flow continues at high velocity along normal channels, while the pelvic flow is diverted up the ascending lumbar and azygos veins to the superior vena cava.
4. The conclusion from abdominal blood-volume measurements that blood is not expelled from the abdomen by raised pressure, taken in conjunction with evidence of rapid flow, implies that the abdominal circulation is not greatly impaired.
5. The maintenance of this circulation is attributed to arteriolar dilatation in the abdomen in response to abdominal distension, and collapse following decompression to failure of the compensatory vasoconstriction.

This work was carried out while I have been in receipt of a Leverhulme Research Scholarship of the Royal College of Physicians. I am also indebted to the Graham Research Fund of the University of London for a grant to cover expenses.

I wish to thank Professor G. R. Cameron, F.R.S., for his help and interest in this work in all its stages. I have also to thank Professor K. J. Franklin for his criticism and some suggestions, Mr F. J. Crew and Mr J. M. Kenney for their technical assistance, Mr V. K. Asta for the illustrations and Mr A. Bligh for the photographs.

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# LOCAL PRODUCTION OF ANTIBODIES

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IT has already been shown (Oakley, Warrack and Batty, 1949) that when alum-precipitated diphtheria or tetanus toxoid is injected subcutaneously into horses, rabbits or guinea-pigs as a secondary stimulus, antibody is produced in the draining lymph-gland, and may, in rabbits, be produced in the injected skin. In this paper we provide evidence that specific antibody may be produced in the skin, fat or voluntary muscle of rabbits and guinea-pigs after a single secondary injection of alum-precipitated diphtheria or tetanus toxoid into those tissues.

## EXPERIMENTAL METHODS

The methods used were similar to those previously reported by Oakley *et al.* Animals were primarily stimulated with a mixture of diphtheria and tetanus alum-precipitated toxoids (diphtheria A.P.T., 60 Lf. per ml.; tetanus A.P.T., 55 Lf. per ml.); at some time over a month later one or other or both antigens were injected into chosen tissues as secondary stimuli. At convenient times thereafter the animals were bled out from the carotid artery, the injected and some uninjected tissues collected, ground with quartz and a suitable volume of 1 per cent. saline—usually 10-20 ml.—and centrifuged. Supernatant and serum were tested for antibodies to both antigens. From the values so obtained the antitoxin ratio, *i.e.* the ratio between the units of tetanus antitoxin per ml. and the units of diphtheria antitoxin per ml. in tissues or their extracts, was determined for each tissue and compared with the antitoxin ratio for the serum. If in such extracts the antitoxin ratio was higher than the ratio in the serum it was assumed that tetanus antitoxin had been manufactured or stored in the tissue extracted; conversely if the serum antitoxin ratio was higher than that of the tissue extract, diphtheria antitoxin had been made or stored in the tissue examined. The decision between manufacture and storage will naturally depend on the tissue involved. For reasons that will appear later we do not consider antitoxin ratios significantly different unless one is at least double the other.

## EXPERIMENTAL RESULTS

### *Significance of differences in antitoxin ratios*

Oakley *et al.* pointed out the considerable effect of errors of testing on estimated antitoxin ratios and considered that only large differences were significant. Examination of their figures shows that for tissues in which the antitoxin ratios might be expected to be equal to those in the serum, the values obtained are not likely to differ from one

TABLE I

*Antitoxin concentrations and antitoxin ratios (tetanus : diphtheria) for rabbit 47. Both hind legs injected with a mixture of equal parts of diphtheria and tetanus A.P.T. as a secondary stimulus. To show distribution of antitoxin ratios when the animal was killed 10 days later*

| Structure extracted | Tetanus antitoxin per ml. extract | Diphtheria antitoxin per ml. extract | Antitoxin ratio | Antitoxin ratio of extract / *Antitoxin ratio* of serum |
|---|---|---|---|---|
| Left foot, skin | 0·17 | 0·077 | 2·2 | 1·1 |
| Right foot, skin | 0·18 | 0·115 | 1·6 | 0·8 |
| Left interscapular fat | 0·475 | 0·31 | 1·5 | 0·75 |
| Right interscapular fat | 0·65 | 0·43 | 1·5 | 0·75 |
| *Omental fat* | *0·027* | *0·0125* | *2·2* | *1·1* |
| Left popliteal gland | 0·165 | 0·10 | 1·7 | 0·85 |
| Right popliteal gland | 0·15 | 0·077 | 2·0 | 1·0 |
| Liver | 0·65 | 0·32 | 2·0 | 1·0 |
| Spleen | 0·24 | 0·15 | 1·6 | 0·80 |
| Left kidney | 0·9 | 0·5 | 1·8 | 0·90 |
| Right kidney | 0·85 | 0·52 | 1·6 | 0·80 |
| Serum | *25·0* | *12·5* | 2·0 | ... |

Assuming a log. normal distribution, the limits for the figures in column 5 (P = 0·95) are 0·64 to 1·23.

TABLE II

*Antitoxin ratios for two rabbits. Numerous organs injected with a mixture of diphtheria and tetanus A.P.T. as a secondary stimulus. To show distribution of antitoxin ratios when the animals were killed 9 and 10 days later*

| Structure extracted | Rabbit 39 | Rabbit 70 |
|---|---|---|
| | Antitoxin ratio | Antitoxin ratio |
| Liver | 1·2 | 1·27 |
| Spleen | 1·27 | 1·33 |
| Left kidney | ... | 1·07 |
| Right kidney | 0·85 | 0·80 |
| Omental fat | 1·32 | 0·67 |
| Left perirenal fat | 1·73 | 0·73 |
| Left broad-ligament fat | 1·27 | 0·63 |
| Left interscapular fat | 1·1 | 1·13 |
| Right interscapular fat | ... | 0·60 |
| Left thigh muscle | 1·1 * | 1·27 |
| Right thigh muscle | 0·84 | 1·27 |
| Diaphragm | ... | 1·20 |
| Left bone-marrow (tibia) † | 1·0 | 1·13 |
| Right bone-marrow (tibia) ‡ | 0·82 | 0·93 |
| Serum | 1·0 | 1·0 |

* Not injected. † Injected with tetanus A.P.T. ‡ Injected with diphtheria A.P.T.

For convenience of comparison, the figures in this and subsequent tables are adjusted to give a serum : antitoxin ratio of 1. Assuming a log. normal distribution, the limits for the antitoxin ratios of both rabbits combined (P = 0·95) are 0·64 to 1·67. Antitoxin ratios of 2 or more will then be evidence of local production of tetanus antitoxin; antitoxin ratios of 0·5 or less will be evidence of local production of diphtheria antitoxin.

another by a factor greater than 2 : 1 (P = 0·05). We have confirmed this figure in two different types of experiment. In the first, rabbits are secondarily stimulated by the injection of mixtures of equal parts of diphtheria and tetanus A.P.T. into both foot pads ; in the second and practically more important experiment, rabbits are secondarily stimulated by the injection of similar mixtures of both antigens into a number of different structures. In both instances it seems reasonable to believe that all the tissues will be influenced in the same proportions by the two antigens and that, whether antibody is produced or not, the antitoxin ratios of all structures including the serum should be the same. Tables I and II show the antitoxin ratios found in the structures of rabbits killed nine or ten days after experiments of both types, and support the view that antitoxin ratios differing by a factor greater than 2 : 1 are significantly different.

*Typical experiment.* A healthy female rabbit (no. 173), wt. 3·30 kg., was primarily stimulated with 0·4 ml. of a mixture of equal parts of diphtheria and tetanus A.P.T. injected subcutaneously into the left flank. One month later the rabbit was anæsthetised with intravenous Nembutal supplemented with ether and the abdomen opened. The liver was injected with 0·2 ml. diphtheria A.P.T. ; the upper pole of the spleen with 0·2 ml. tetanus A.P.T. and the lower with 0·2 ml. diphtheria A.P.T. In injecting liver and spleen care was taken to vary the direction of the needle several times, so as to reduce the risk of intraperitoneal hæmorrhage. The abdomen was then closed in two layers, 0·2 ml. diphtheria A.P.T. injected intracutaneously into the skin of the left flank and 0·2 ml. tetanus A.P.T. similarly into the right flank, and the animal allowed to recover. Ten days later it was bled out and the injected areas and some uninjected areas collected, and the extracts titrated for diphtheria and tetanus antitoxins. (The white nodules formed make the injection areas fairly easy to find.)

*Results.* Table III shows that the antitoxin values for the liver and spleen of this rabbit differed little from the ratio for the serum, whether they were injected with tetanus or diphtheria A.P.T., or with nothing at all ; there was no evidence of localised antibody production in these organs. Skin, on the other hand, if injected with diphtheria A.P.T., showed evidence of local excess of diphtheria antitoxin ; if it were injected with tetanus A.P.T., tetanus antitoxin appeared to be present in excess. In default of any other source from which this excess antitoxin could have been derived, we conclude that this specific antitoxin is produced in the injected skin. It is noteworthy that antitoxin was produced locally in the skin after a single secondary injection of A.P.T. ; there is no evidence that a pre-formed granuloma is necessary, as we had previously supposed. Our opinion that antitoxin is produced in other injected tissues (*vide infra*) is based on the same argument.

Table III also shows the antitoxin ratios for rabbit 178, which had been secondarily injected in liver, spleen, skin and perirenal fat. The figures confirm those for rabbit 173, but show also that specific antibody is produced in injected perirenal fat. We had been stimulated to try perirenal fat by the observation of Bjørneboe *et al.* (1947) that high concentrations of antibody can be demonstrated in the perirenal fat of rabbits injected with pneumococcal polysaccharides; the finding that it can produce antibody led us to try other fat areas. Thus in some rabbits we injected diphtheria or tetanus A.P.T. as secondary stimulus into gastric omental fat, testicular omentum, broad-ligament or interscapular fat. In the gastric omentum one antigen was injected near the spleen, the other distant from it; in the other sites the paired structure of the fat made it possible to inject one antigen on each side. Table IV gives antitoxin ratios for two rabbits. Perirenal

TABLE III

*Antitoxin ratios for two rabbits injected in various organs with diphtheria or tetanus A.P.T. as a secondary stimulus and killed* 10 *days later. To show evidence of antibody production in skin and perirenal fat*

| Structure injected | A.P.T. injected | | | A.P.T. injected | | |
|---|---|---|---|---|---|---|
| | Tetanus | Diphtheria | None | Tetanus | Diphtheria | None |
| Liver | ... | 0·51 | 1·0 | 1·68 | ... | 1·16 |
| Spleen | 1·7 | 2·05 | ... | 1·10 | 0·87 | ... |
| Skin | 17·4 | 0·105 | ... | 8·42 | 0·16 | ... |
| *Perirenal fat* | ... | ... | ... | *17·6* | *0·21* | ... |
| Serum | 1·0 | | | 1·0 | | |
| Rabbit no. | 173 | | | 178 | | |

TABLE IV

*Antitoxin ratios for two rabbits injected in various organs with diphtheria or tetanus A.P.T. as a secondary stimulus and killed* 10 *days later. To show evidence of antibody production in fat*

| Structure injected | A.P.T. injected | | | A.P.T. injected | | |
|---|---|---|---|---|---|---|
| | Tetanus | Diphtheria | None | Tetanus | Diphtheria | None |
| Liver | ... | 0·87 | 0·96 | 1·07 | ... | 1·1 |
| Spleen | 1·1 | ... | ... | ... | 0·93 | 1·0 |
| Kidney | 1·0 | 0·70 | ... | 1·07 | 0·79 | ... |
| Perirenal fat | 11·0 | <0·043 | ... | 16·4 | 0·071 | ... |
| Testicular omentum | 6·1 | 0·083 | ... | 5·7 | 0·093 | ... |
| Serum | 1·0 | | | 1·0 | | |
| Rabbit no. | 193 ♂ | | | 199 ♂ | | |

and testicular omental fat show good evidence of local antibody production. liver, spleen and kidney show none. In these rabbits only one antigen was injected into any unpaired organ, to avoid the objection that mixing of the antigens might occur, and that local production of antibody might thus be masked.

Table V gives antitoxin ratios for two female rabbits in which perirenal, omental, interscapular and broad ligament fat were secondarily injected with one or other antigen, as well as spleen, extensor muscle of thigh and bone-marrow. This last was approached through a burr hole in the inner face of the upper end of the tibia. While spleen and bone marrow show no evidence of antitoxin production, all the fat areas injected produce antitoxin locally. Table VI, which gives antitoxin ratios for two male rabbits, shows (*a*) that bone-marrow and lung (injected intra-tracheally) fail to show evidence of antibody production, (*b*) that all fat areas injected, as well as thigh muscle and perhaps popliteal gland—injected directly after exposure through an incision in the popliteal fossa—produce antibody. The results for skin in rabbit 12 are particularly interesting as they show that both intracutaneous and subcutaneous secondary stimulation lead to local production of antibody.

TABLE V

*Antitoxin ratios for two rabbits injected in various organs with diphtheria or tetanus A.P.T. as a secondary stimulus and killed 10 days later. To show evidence of antibody production in fat and voluntary muscle*

| Structure injected | A 1 T injected | | A P T injected | |
|---|---|---|---|---|
| | Tetanus | Diphtheria | Tetanus | Diphtheria |
| Spleen | 1 4 | 0 83 | 1 1 | 1 1 |
| Bone marrow | 0 86 | 1 3 | 1 0 | 1 0 |
| Perirenal fat | 5 0 | 0 042 | 25 0 | 0 033 |
| Omental fat | 2 2 | 0 12 | 2 1 | 0 18 |
| Broad ligament fat | 1 6 | 0 17 | 8 6 | 0 20 |
| Interscapular fat | 2 0 | <0 2 | 14 0 | 0 10 |
| Thigh muscle | 6 9 | 0 036 | 62 0 | 0 043 |
| Serum | 1 0 | | 1 0 | |
| Rabbit no | 33 ♀ | | 165 ♀ | |

Altogether we have used 42 rabbits in these and similar experiments. The results have been extremely consistent: antitoxin is produced locally in secondarily-injected skin, voluntary muscle or fat, in injected liver, spleen, kidney and bone marrow there is no evidence of antitoxin production. As judged by our methods, local production of antitoxin can occur in skin, voluntary muscle or fat as early as three days after a secondary injection. It has unfortunately never been possible to obtain sufficiently high titres of tissue antitoxin in primarily-stimulated animals for this problem to be investigated in them.

TABLE VI

*Antitoxin ratios for two rabbits injected in various organs with diphtheria or tetanus A.P.T. as a secondary stimulus and killed* 10 *days later. To show evidence of antibody production in skin, fat and voluntary muscle*

| Structure injected | A.P.T. injected | | A.P.T. injected | |
|---|---|---|---|---|
| | Tetanus | Diphtheria | Tetanus | Diphtheria |
| Bone-marrow . . . | 1·0 | 1·2 | 1·0 | 0·88 |
| Lung . . . . . | ... | ... | 1·1 | ... |
| Skin (intracutaneous) . | 1·7 | 0·044 | ... | ... |
| Skin (subcutaneous) . . | 18·0 | 0·12 | ... | ... |
| Perirenal fat . . . | 4·1 | 0·061 | ... | ... |
| Omental fat . . . | 9·4 | 0·67 | ... | ... |
| Testicular omentum . . | 4·3 | 0·13 | ... | ... |
| Interscapular fat . . | 15·0 | 0·072 | 2·8 | 0·58 |
| Thigh muscle . . . | 53·0 | 0·033 | 8·1 | 0·31 |
| Popliteal gland . . | ... | ... | 1·3 | 0·54 |
| Serum . . . . | 1·0 | | 1·0 | |
| Rabbit no. . . . | 12 | | 50 | |

### *Local production of antitoxin in guinea-pigs*

We have injected diphtheria or tetanus A.P.T. into various organs and tissues of six adult guinea-pigs as a secondary stimulus; examination of the antitoxin ratios of organ and tissue extracts ten days after injection shows that antitoxin may be produced in skin, fat and voluntary muscle (table VII gives the figures for three of the guinea-pigs: the results for the others were very similar). No evidence was obtained of antitoxin production in liver, spleen or kidney.

### *The history of antitoxin production in the skin*

As secondarily-injected skin had consistently produced antitoxin we thought it worth while to follow the history of antitoxin production in it, especially as it seemed likely that the antitoxin ratios might be fairly comparable. We therefore clipped the hair from the back of a number of primarily-stimulated rabbits and divided the exposed skin into a number of symmetrically disposed equal squares. Each day a square was selected at random on one side of the rabbit and injected intracutaneously with 0·2 ml. diphtheria or tetanus A.P.T., chosen by the use of random numbers. The other antigen was injected into the corresponding square on the other side, and the process repeated daily till all available squares were used up. The animal was then

TABLE VII

*Antitoxin ratios for three guinea-pigs injected in various organs with diphtheria or tetanus A.P.T. as a secondary stimulus and killed 10 days later. To show evidence of antibody production in skin and fat (guinea-pigs 176 and 750), and voluntary muscle (all three guinea-pigs)*

| Structure extracted | Guinea-pig 106 | | Guinea-pig 176 | | Guinea pig 750 | |
|---|---|---|---|---|---|---|
| | A P T injected | | A P.T injected | | A P.T. injected | |
| | Tetanus | Diphtheria | Tetanus | Diphtheria | Tetanus | Diphtheria |
| Liver . . . | ... | 1·0 | 1·2 | ... | ... | 1·0 |
| Spleen . . . | 0·79 | ... | ... | 1·2 | 0·93 | ... |
| Kidney . . . | 0·79 | 1·2 | ... | ... | 0·96 | ... |
| Skin . . . . | 0·75 | 0·57 | 2·5 | 0·15 | 1·3 | 0·40 |
| Thigh muscle . . | 2·8 | 0·039 | 2·1 | 0·12 | 6·4 | 0·11 |
| Perirenal fat . . | ... | ... | 3·0 | 0·071 | ... | <0·13 |
| Broad-ligament fat . | 1·4 | 0·82 | 1·5 | 0·087 | ... | ... |
| Serum . . . | 1·0 | | 1·0 | | 1·0 | |

bled out and the thickened area in each square excised, extracted and tested in the usual way. By this means we could obtain antitoxin ratios for rabbit skin secondarily injected from one to ten or more days previously. Fig. 1 shows the relationship between the log. antitoxin value of such pieces of skin and the time after injection. In those injected with either antigen the antitoxin ratio for the first two days is identical with that in the serum. Thereafter the antitoxin ratio for skin injected with either antigen diverges progressively from the ratio in the serum, the points relating log. antitoxin ratio to time lying reasonably close to a straight line.

Assuming that the heterologous antibody present in the skin samples is derived from the serum or from blood left in the capillaries and that this is fairly constant all over the dorsal skin, we conclude that from the third to the tenth day after injection antitoxin increases logarithmically in secondarily-injected skin, and suggest that the cells producing it are increasing in the same way.

We therefore repeated the experiment so that animals were injected (1) on 20 successive or (2) on 20 alternate days. Fig. 2 shows the result of the first experiment. In the pieces of skin injected with tetanus antitoxin no rise in antitoxin ratio occurs till the fourth day: apart from one break there is a steady logarithmic rise till the twelfth day followed by a slow fall; the whole resembles very closely the early stages of a secondary response as measured in circulating antitoxin. Fig. 3 plots log. antitoxin ratio against time for 39 days

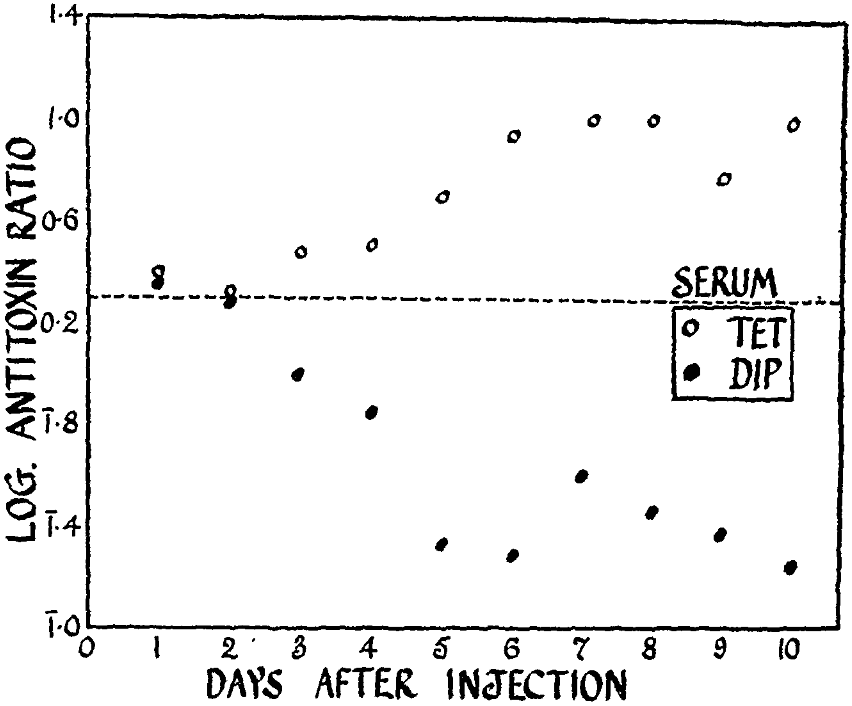

FIG. 1.—Log. antitoxin ratios for injected skin areas from rabbit injected daily for 10 days with diphtheria and tetanus A.P.T., plotted against length of time after injection. Open circles = areas injected with tetanus A.P.T.; closed circles = areas injected with diphtheria A.P.T.

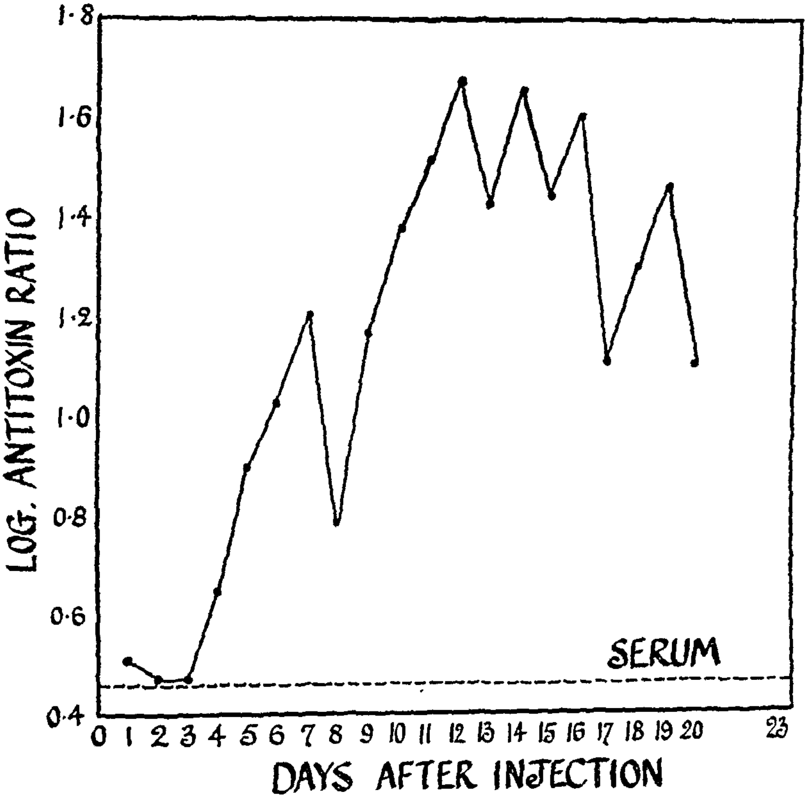

FIG. 2.—Log. antitoxin ratios for skin areas injected with tetanus A.P.T. from rabbit injected daily for 20 days with diphtheria and tetanus A.P.T., plotted against length of time after injection.

(expt. 2). The resemblance to the secondary response curve in circulating antitoxin is remarkably close, both in shape and in relationship to time, and we suggest that the form of the secondary response curve in serum is only a reflection of the activity of the antibody-producing centres, affected only by the fact that antibody is destroyed at a rate proportional to its concentration. Unfortunately we could not obtain antitoxin ratios for the pieces of skin injected with diphtheria A.P.T. in either of these rabbits, as the tetanus antitoxin content of the pieces was below the lowest level at which it was possible to obtain a positive reaction on test.

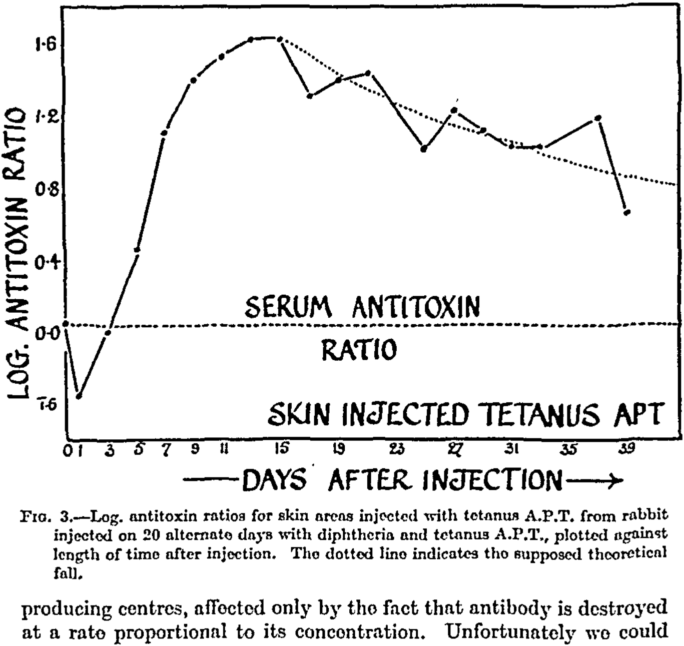

FIG. 3.—Log. antitoxin ratios for skin areas injected with tetanus A.P.T. from rabbit injected on 20 alternate days with diphtheria and tetanus A.P.T., plotted against length of time after injection. The dotted line indicates the supposed theoretical fall.

### *Failure of horses to produce antitoxin in skin*

Oakley *et al.* have shown in a few horses that the subcutaneous injection of a single dose of diphtheria or tetanus A.P.T. as a secondary stimulus seldom leads to local antibody production, though two injections in the same area may stimulate local production. We have continued these experiments in a further ten horses with much the same result, as shown in table VIII. Unless there is some fundamental defect in our experiments, it seems clear that horses differ markedly from rabbits in their incapacity to produce antibody locally in the skin. The results for two horses injected with toxoids in the skin are set out for comparison.

TABLE VIII

*Antitoxin ratios in injected skin of horses injected with diphtheria antigen (right hind leg) and tetanus antigen (left hind leg) as secondary stimuli. No evidence of antitoxin production in skin* 10 *days later*

| Horse | Secondary stimuli | Antitoxin ratios for | | |
|---|---|---|---|---|
| | | left skin | right skin | serum |
| 5016 | Diphtheria and tetanus A.P.T. | 1·6 | 0·95 | 1·0 |
| 5588 | ,, ,, | 1·1 | 1·1 | 1·0 |
| 5718 | ,, ,, | 1·1 | 1·4 | 1·0 |
| 5918 | ,, ,, | 1·3 | 1·2 | 1·0 |
| 5919 | ,, ,, | 0·91 | 0·71 | 1·0 |
| 6047 | ,, ,, | 1·4 | 1·0 | 1·0 |
| 6102 | ,, ,, | 1·5 | 1·6 | 1·0 |
| 6335 | ,, ,, | 1·3 | 1·1 | 1·0 |
| 5863 | Diphtheria and tetanus toxoid | 1·2 | 1·1 | 1·0 |
| 6067 | ,, ,, | 0·96 | 0·93 | 1·0 |

Two other horses, one injected with diphtheria and *Cl. welchii* α-A.P.T. and another injected with *Cl. welchii* β-A.P.T. as secondary stimuli, which also failed to show any sign of antitoxin production in the skin, are for convenience omitted.

## DISCUSSION

Until recently, it was the general opinion, though there was little evidence to support it, that antibodies were produced generally in the reticulo-endothelial tissue, and particularly in structures rich in this tissue, such as the spleen, liver and bone-marrow. Ehrich and his school (Ehrich and Harris, 1942 ; Harris *et al.*, 1945) first drew attention to the lymphocyte as a possible source of antibody, though more recent work (Ehrich *et al.*, 1949) has caused them to support Bjørneboe and Gormsen (1943), Bjørneboe *et al.* (1947) and Fagraeus (1946, 1947, 1948) in making out a case for the plasma cell. However, as Fagraeus believes that plasma cells are derived from reticular cells, this may be regarded as support for the reticulo-endothelial theory. The results of our experiments have caused us no little surprise, since, though we could consistently obtain evidence for antibody production in the skin, fat and voluntary muscle in rabbits, we could get no such evidence for the liver, spleen, kidney or bone-marrow. It might be argued that the liver, spleen and bone-marrow are highly vascular and that antibody formed there might be removed as fast as it was produced. But surely this applies to muscle as well ? A more forceful argument is that alum-precipitated toxoids can hardly be injected into liver, spleen or bone marrow without disrupting them, whereas injections into skin, fat and voluntary muscle might split up these tissues without severely affecting their functions. We do not, of course, necessarily believe that antitoxins are formed by skin cells, fat cells or muscle cells; they may be produced by connective tissue cells, or the cells of the granuloma that develops rapidly round the injected A.P.T. In the hope of attacking this problem we have collected a large amount of material from injected areas for histological examination.

The idea that antibody might be produced in injected skin is not new. It was put forward by Cannon and Sullivan (1931-32) for rabbits injected with killed bacteria and, with much more convincing experiments, by Ørskov and Andersen (1938), who claimed that the rapid fall in the amount of virus recoverable from the skin of rabbits infected with vaccinia was due to local production of antibody; they showed that virus-neutralising antibody could be recovered from the skin four days after infection. The literature on local production of antibody in skin and some other tissues is thoroughly reviewed by Burnet and Fenner (1949). As far as we know, no evidence has previously been offered for the production of antibody in fat or voluntary muscle.

On the other hand, the position of the liver as the main source of plasma protein and therefore by implication of at least some antitoxic protein, has recently been challenged by Cheng (1949), who found that severe damage to the liver, produced in a variety of ways, made little difference to the rate at which plasma protein was synthesised in rats. He suggests, among other possibilities, that cells in general may be capable of synthesising plasma protein, and points out the remarkable protein-synthesising power of connective tissue cells and —even more striking—of such specialised structures as muscle cells. It seems possible that experiments on such relatively accessible structures as skin and muscle might throw some light on this problem.

The alteration in antitoxin ratios with time in the skin of rabbits raises some interesting questions, for if the basic assumptions are accepted, the natural interpretation of our results is that after the first two or three days following injection, antitoxin-producing cells increase logarithmically for about ten days, remain steady in number or effectiveness for a short time and then slowly fall. There are clearly two possibilities. (1) Numbers of cells only may be involved, in which case we must conclude that after the response has reached its peak at the tenth to the twelfth day after secondary stimulation, the division of antitoxin-producing cells goes on at a slower rate, though all the cells produced are about equally efficient. (2) Alternatively we might suppose that as antibody-producing cells divide, the reproduction of antibody-producing " centres " in the cell becomes less and less accurate, so that after a time the amount of protein possessing demonstrable antibody activity becomes less and less, although new " centres " are constantly being set up in other cells. It is difficult to see how experiment can settle this point.

## SUMMARY

After a single secondary injection of diphtheria and tetanus alum-precipitated toxoid into the skin, fat or voluntary muscle of rabbits or guinea-pigs, specific antitoxin may be produced in the injected tissues. No evidence of antibody production can be obtained in the liver, spleen, kidney or bone-marrow similarly injected.

Examination of the change with time in the antitoxin ratios in the skin of rabbits injected with tetanus and diphtheria A.P.T. suggests that the changes in the circulating antibody after a secondary stimulus are in part a reflection of activity in the antibody-producing areas.

No evidence has been obtained that a single subcutaneous secondary injection of diphtheria or tetanus A.P.T. will elicit local production of antitoxin in the horse.

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# THE ACRA TEST AS A MEANS OF ESTIMATING HYALURONIDASE, DEOXYRIBONUCLEASE AND THEIR ANTIBODIES

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BURNET (1948) devised the ACRA test (acid-congo red-alcohol) for the demonstration of hyaluronidase in culture filtrates of *Cl. welchii* and *Staphylococcus aureus*. The test depends on the fact that mixtures of human synovial fluid and aqueous congo red will, under suitable conditions, form a compact blue lenticular "blob" when a drop of the mixture is allowed to fall into acid alcohol (1 per cent. concentrated HCl in 70 per cent. alcohol). Progressive reduction of the amount of hyaluronic acid in the mixture, either by dilution or by enzymatic digestion with hyaluronidase, leads to progressive changes in the blob formed in the test; it becomes less cohesive, with less distinct edges; then it becomes annular, and at sufficient dilution the drop of synovial fluid and congo red spreads freely into the acid alcohol. Specific antihyaluronidases will inhibit the digestion of synovial fluid by hyaluronidase and may be calibrated for this property. Burnet has also applied the method to the detection of mucinases and anti-mucinases.

We have found that with suitable modifications the test can be used to estimate hyaluronidase and deoxyribonuclease and to calibrate sera for antihyaluronidase and antideoxyribonuclease with considerable accuracy.

## EXPERIMENTAL OBSERVATIONS

### *Factors affecting the test for hyaluronidase*

*The substrate.* For most of our work we have used horse synovial fluid; there is good reason to suppose that other synovial fluids would work equally well. Different samples of horse synovial fluid, however, differed markedly in their properties; some produced a good blob in the ACRA test at a dilution of 1 in 500, others spread freely, even undiluted. It was therefore always necessary to test the synovial fluid on every occasion before use; in all tests about eight indicating doses of substrate were used. It can readily be shown (table I) that the standard indicating dose and test dose of filtrates depend on the number of indicating doses of substrate present (*cf.* Burnet). The keeping properties of horse synovial fluid also varied greatly; some samples would keep for weeks in the cold box, then suddenly, though apparently uninfected, become useless; others would spread undiluted after only a few days' cold storage.

Horse synovial fluid precipitated with saturated alcoholic potassium acetate has proved satisfactory, a good blob being produced at a concentration of 0·05 per cent. of the dried precipitate. Hyaluronic acid prepared from the umbilical cord by Hadidian and Pirie's method (1948) and hyaluronic acid obtained from commercial houses proved useless for this test.

TABLE I

*Relation between synovial fluid concentration in ACRA tests and the standard indicating dose and test dose of a* Cl. welchii *filtrate*

| Synovial fluid used | Standard indicating dose of filtrate | Test dose of filtrate |
|---|---|---|
| 0·5 ml. 1/2 | 0·004 ml. | 0·51 ml. |
| „ 1/4 | 0·002 ml. | 0·51 ml. |
| * „ 1/8 | 0·002 ml. | 0·46 ml. |
| „ 1/16 | 0·001 ml. | 0·38 ml. |

* Amount used in tests.

The synovial fluid contained 128 indicating doses per ml.

*The diluting fluid.* The most satisfactory diluent was borate-buffer-saline *p*H8 (B.B.S.), which we have used throughout; 1 per cent. saline was almost as good; *M*/20 phosphate-buffer-saline *p*H 7 was unsatisfactory; the blobs dispersed readily and the results were not so reproducible as with other diluents.

*The concentration of congo red.* The final concentration of congo red in the tests has a decisive effect on the results. In general, the lower the concentration of substrate, the higher the concentration of congo red necessary to form a blob-producing mixture; for any constant concentration of substrate, increasing concentrations of congo red produce a steady increase in the coherence of the blob formed. Table II shows the result obtained when a constant amount and concentration of substrate is treated with constant volumes of varying concentrations of congo red. The effect of congo red is not due to the increasing concentration of congo red altering the viscosity of the mixtures, for the viscosity of all the mixtures in table II (relative to water) was practically constant, though the concentration of congo red varied from 0·06 to 2 per cent.

TABLE II

*Effect of congo-red concentration on the pattern obtained in the ACRA test (2·0 ml. diluted synovial fluid plus 0·4 ml. congo-red dilution)*

| Synovial fluid used | Concentration of congo red used | | | | | |
|---|---|---|---|---|---|---|
| | 0·06 | 0·13 | 0·25 | 0·5 | 1·0 | 2·0 per cent. |
| 1 | spread | annulus | blob | blob | blob | blob |
| 2 | „ | spread | „ | „ | „ | „ |
| 3 | „ | „ | „ | „ | „ | „ |

*The diameter and height of the capillary nozzle.* The greater the diameter of the capillary, the larger the drop formed by it and the greater the kinetic energy with which it strikes the acid alcohol. A similar effect is produced if the height of the capillary nozzle above the acid alcohol is increased. If either the diameter of the capillary or the height from which the drop falls is too great, a drop which should form a blob may be flattened out into an apparent spread on the bottom of the petri dish by the force of impact. We have usually used a pasteur pipette of 1 mm. internal diameter and dropped the liquid from 1 cm. above the acid alcohol.

*The depth of the acid-alcohol layer.* Reducing the depth of the acid-alcohol layer has much the same effect as allowing the drops to fall from too great a height. To avoid variations in the depth of the acid alcohol, the petri dish is levelled up before use, with a piece of white filter paper underneath it to show up the blobs, and filled to a measured depth of at least 4·5 mm.

### *Tests for hyaluronidase and antihyaluronidase*

*Preliminary test for substrate.* Prepare doubling dilutions of horse synovial fluid in B.B.S. in 0·5 ml. volumes in Lambeth tubes, add 1·5 ml. B.B.S. to each, mix by inversion over non-absorbent paper and incubate in a water-bath at 37° C. for one hour. Remove the tests from the bath and cool them rapidly in ice-cold water; add to each tube 0·4 ml. of 0·5 per cent. congo red, mix by inversion, allow to stand for five minutes and then test as below.

Level a petri dish of convenient size and fill with acid alcohol to a measured depth of at least 4·5 mm. Remove a suitable quantity of fluid from each tube with a pasteur pipette (internal diameter 1 mm.) and allow a drop to fall from a height of 1 cm. into the acid alcohol. The results are read directly from the appearance of the patterns formed. For our purpose the tube containing the smallest amount of substrate giving a good cohesive blob is taken as the end-point, and is said to contain one indicating dose of substrate. For testing hyaluronidases and antisera eight indicating doses of substrate are used.

*Test for* Cl. welchii *hyaluronidase.* Prepare doubling dilutions in B.B.S. in 1·0 ml. volumes of fluid to be tested for hyaluronidase, add to each 0·5 ml. B.B.S. and 0·5 ml. horse synovial fluid diluted in B.B.S. to contain 8 indicating doses. Then proceed exactly as in tests for substrate. Tubes containing high concentrations of hyaluronidase produce a complete spread in the test; the end-point is taken as the tube containing the least amount of test substance from which the drop spreads freely. This is said to contain one standard indicating dose; different observers obtain consistent results with very little practice. Controls using buffer and substrate only were always included in the test.

*Results.* A number of filtrates were obtained from strains of *Cl. welchii* grown for 5-18 hours in meat broth plus a concentrated papain digest of horse muscle and a large amount of cooked horse-meat particles. Table III shows the results obtained in ACRA tests

TABLE III

*Production of hyaluronidase by* Cl. welchii

| Type | No. of strains examined | Positive | Negative |
|---|---|---|---|
| A | 14 | 7 | 7 |
| B | 14 | 14 | 0 |
| C | 9 | 0 | 9 |
| D | 19 | 4 | 15 |
| E | 6 | 0 | 6 |
| F | 6 | 0 | 6 |

for hyaluronidase ; filtrates giving positive results usually contained 1000-2000 standard indicating doses per ml.

The results for *Cl. welchii* type A agree very well with those obtained by McClean, Rogers and Williams (1943), Evans (1945) and Kass, Lichstein and Waisbren (1945), who found that 33-52 per cent. of the strains examined produced hyaluronidase. Chain and Duthie (1940) state that *Cl. welchii* types A and B produce hyaluronidase, but that types C and D do not ; the number of strains examined is not stated. In our hands all type-B and some type-D strains have given positive results ; the few strains of types C, E and F were consistently negative.

Since McClean and Hale (1941) and Rogers (1945) emphasise the importance of the addition of hyaluronic acid to the medium for the enhancement of hyaluronidase production by *Cl. welchii* strains, one *Cl. welchii* type-A, one type-B, two type-C, two type-D, two type-E and two type-F strains were grown for 5, 24 and 48 hours in the meat-broth medium referred to above plus 0·15 per cent. hyaluronate, and compared with controls grown in the meat-broth medium alone. There was no enhancement of production by the positive type-A and -B strains, and no evidence of any hyaluronidase production by the -C, -D, -E or -F strains. This result may be due to our use of media different from those used by other workers : it is possible that our basic medium contained more than the optimum amount of hyaluronate, unlike the casein hydrolysate medium used by Rogers, the peptone plus beef-extract medium used by Evans or the peptone medium used by McClean and Hale.

Kass *et al.* state that *Cl. welchii* strains lose their capacity to produce hyaluronidase in meat-broth medium if they are repeatedly subcultured, even when hyaluronic acid is added to the medium. Some of our positive type-A and -B strains had been subcultured very many times, while one type-C, four type-E and six type-F strains had been recently isolated but failed to produce hyaluronidase even when hyaluronic acid was added to the medium. It seems reasonable to conclude that the strains of type C, E and F examined by us do not produce hyaluronidase.

*Test for antihyaluronidase.* EX 902 was chosen as standard and given an arbitrary antihyaluronidase value of 200 units ; tests were carried out at one unit, at which level the test dose contained about 250 standard indicating doses. The test dose was determined as follows :—

To amounts of filtrate differing by 10 per cent. add one unit of serum diluted in B.B.S. and make the volume up to 1·5 ml. with B.B.S. Mix the tubes by inversion and allow them to stand at room temperature for 30 mins. ; add 0·5 ml. synovial fluid diluted to contain 8 indicating doses and proceed as in hyaluronidase testing. Take as test dose the minimum amount of filtrate which, when mixed with one unit of antitoxin, just produces a good spread in the ACRA test. This test dose is then used to calibrate other sera in the usual way.

*Results.* The majority of sera gave consistent answers ; one only (RX 5457), tested against the same filtrate, gave a range of answers from 1300 to 2000 : this inconsistency is so far unexplained. Table IV gives the values of a number of sera tested against the hyaluronidase present in filtrates of *Cl. welchii* type-A, type-B and two type-D strains—one producing λ-antigen, the other not (Oakley, Warrack and Warren, 1948). It is clear that the antihyaluronidase values of the sera are independent of the source of the filtrate used, and therefore that the hyaluronidases produced by these type-A, type-B and type-D strains are antigenically equivalent.

TABLE IV

*Comparison of antihyaluronidase values of sera against four different* Cl. welchii *filtrates to show that the same hyaluronidase is present in all*

| Serum | Antihyaluronidase value against filtrate of type | | | |
|---|---|---|---|---|
| | A | B | D | D |
| EX 902 * | 200 | 200 | 200 | 200 |
| EX 1101 | 90 | 80 | 80 | 60 |
| RX 5218 | 250 | 210 | 210 | 240 |
| RX 5457 | 1300-2000 | 1500 | 1100 | 1500 |
| R 5603 | 120 | 90 | 90 | 90 |
| R 5609 | 200 | 180 | 170 | 170 |
| R 6423 | 35 | 40 | 30 | 40 |
| R 7483 | <6 | <6 | <6 | <6 |
| R 8531 | 70 | 80 | 85 | 95 |
| RR 3518 | <6 | <6 | <6 | <6 |
| RR 5959 | 250 | 240 | 280 | 260 |
| LX 251 | 80 | 100 | 70 | 70 |
| GGC 3296 | 80 | 90 | 70 | 70 |
| GWP 160 | 750 | 730 | 770 | 800 |

* Standard.

*Avidity in antihyaluronidase sera.* It was found possible to carry out reasonably accurate antihyaluronidase tests at 1/20 unit : determinations of the avidity of sera were therefore practicable. The test dose of a *Cl. welchii* type-A filtrate was determined at 1, 0·5, 0·25, 0·125 and 0·0625 units ; the results are given in table V.

TABLE V

*Hyaluronidase test dose of a* Cl. welchii *filtrate at levels ranging from 1 to 1/16 unit, to show varying degrees of avidity of sera*

| Serum | Test dose of *Cl. welchii* filtrate in ml. at unit level | | | | |
|---|---|---|---|---|---|
| | 1 | 1/2 | 1/4 | 1/8 | 1/16 |
| EX 902 | 0·62 | 0·51/2 | 0·46/4 | 0·23/8 | <0·13/16 |
| EX 1101 | 0·62 | 0·68/2 | 0·62/4 | <0·42/8 | ... |
| RX 5218 | 0·51 | 0·68/2 | 0·51/4 | 0·34/8 | ... |
| RX 5457 | 0·63 | 0·92/2 | 0·92/4 | 0·84/8 | 0·63/16 |
| R 5609 | 0·62 | 1·0/2 | >1·0/4 | 0·74/8 | ... |

RX 5457, RX 5218, R 5609 and EX 1101 are reasonably avid : EX 902 is very non-avid, its test dose becoming relatively less with increasing dilution of serum.

*Antihyaluronidase in normal horse sera.* *Cl. welchii* antihyaluronidase has been reported in normal sera by Hobby *et al.* (1941) and McClean (1942); we therefore tested a number of fresh and aged normal horse sera for *Cl. welchii* antihyaluronidase at 1/20 unit level. All sera tested at this level had less than 1·5 units; it was impossible to test them below this, as concentrations of serum greater than 1 in 30 themselves caused complete spreading in the ACRA test.

We have also tested normal sera by the less satisfactory method of preparing twofold dilutions of *Cl. welchii* hyaluronidase and adding 1 ml. of serum diluted 1 in 30 to each of them. No antihyaluronidase could be detected in normal horse sera by this method.

*Experiments with partially purified hyaluronic acid.* Partially purified hyaluronic acid was prepared from synovial fluid as follows :— One and one-third volume of ethanol saturated with potassium acetate and cooled to −10° C. was added to one volume of horse synovial fluid, the stringy clot well washed with alcohol and then with ether and dried over phosphorus pentoxide. The white powder so obtained was dissolved in distilled water and 0·5 ml. of a 0·05 per cent. solution used in the tests.

Table VI shows the values of sera in ACRA tests against *Cl. welchii* type-A and type-B filtrates, using synovial fluid and partially purified hyaluronic acid as substrates : the results agree well and leave no doubt that the same enzyme is titrated in all cases.

TABLE VI

*Comparison of antihyaluronidase values of sera against a* Cl. welchii *type-A and a type-B filtrate, using as substrate synovial fluid or hyaluronic acid*

| Serum | Serum values against filtrate of | | | |
|---|---|---|---|---|
| | type A, using as indicator | | type B, using as indicator | |
| | synovial fluid | hyaluronic acid | synovial fluid | hyaluronic acid |
| EX 902 * | 200 | 200 | 200 | 200 |
| EX 1101 | 90 | ... | 80 | 60 |
| RX 5218 | 250 | 250 | 240 | 270 |
| RX 5457 | 1300-2000 | 1650 | 1500 | 2000 |
| R 5603 | 120 | 100 | 90 | 90 |
| R 5609 | 200 | 200 | 180 | 160 |
| R 6423 | 35 | 30 | 40 | 50 |
| R 7577 | <6 | <8 | <6 | <6 |
| R 8531 | 70 | 95 | 80 | 80 |
| RR 3518 | <10 | <8 | <6 | <6 |
| LX 251 | 80 | 80 | 100 | 90 |
| GGC 3296 | 80 | 110 | 90 | 90 |
| GWP 160 | 750 | 610 | 730 | 730 |

* Standard.

*Nomenclature.* The only known toxins of *Cl. welchii* common to types A, B and D are *Cl. welchii* $\alpha$ and $\theta$ toxins. Table VII shows that the *Cl. welchii* antihyaluronidase values of sera bear no relation to their anti-α or anti-θ values, *Cl. welchii* hyaluronidase is antigenically distinct from α and θ toxins, and—by elimination—from the other

TABLE VII

*Comparison of* Cl. welchii *antihyaluronidase values of sera with their anti-α and anti θ values, to show that* Cl. welchii *hyaluronidase is antigenically distinct*

| Serum | Antihyaluronidase value | Anti α value | Anti θ value |
|---|---|---|---|
| EX 902 * | 200 | 200 | 140 |
| EX 1101 | 90 | 20 | 30 |
| RX 5218 | 250 | 3090 | 1100 |
| R 5603 | 120 | 280 | 0·7 |
| R 5609 | 200 | 320 | 16 |
| R 6123 | 35 | 70 | 70 |
| R 7843 | <6 | 170 | 130 |
| RR 3518 | <6 | 220 | 32 |
| LX 251 | 80 | 160 | 0·2 |
| GGC 3296 | 80 | 370 | 40 |
| GWP 160 | 750 | 4500 | 16 |

* Standard

known toxins of *Cl. welchii*. We find it convenient to call it *Cl. welchii* Mu-antigen (μ-antigen).

### *Factors affecting the test for deoxyribonuclease*

Most of the factors affecting the test for hyaluronidase are equally important in deoxyribonuclease tests: a few additional points are referred to here

*The substrate.* We have used throughout sodium deoxyribonucleate prepared from horse spleen by the method of Gulland, Jordan and Threlfall (1947) (see Warrack, Bidwell and Oakley, 1951) It was dissolved in M/40 veronal buffer, *p*H 7·5, containing 0 003 *M* magnesium (McCarty, 1945 46) by gentle shaking in a water-bath at 37° C. Commercial deoxyribonucleate proved unsatisfactory for ACRA tests.

*Diluents.* Veronal buffer was used for diluting substrate, 1·5 per cent. neopeptone in 1 per cent. saline for diluting filtrates and sera.

## *ACRA tests for deoxyribonuclease and antideoxyribonuclease*

*Test for substrate.* Dissolve sodium deoxyribonucleate in veronal buffer as above to give a convenient concentration. From this solution prepare two fold dilutions in 0·5 ml. volumes of veronal buffer and add 1·5 ml. of 1·5 per cent. neopeptone to each tube. Mix by inversion and incubate in a water-bath at 37° C. for one hour. Subsequent testing is exactly as in the ACRA tests for synovial fluid. The blob formed in the test is rather smaller than that produced by synovial fluid, and tends to break up more readily, though if it contains sufficient deoxyribonucleate it always retains some structure The batch of sodium deoxyribonucleate used in our tests gave a good viscous blob at 0·13 per cent., a fair blob at 0 065 per cent, and spread at 0 033 per cent. A concentration of 0 13 per cent. was therefore used in our tests.

*Test for* Cl. welchii *deoxyribonuclease.* Filtrates can be tested for deoxyribonuclease by estimation of the maximum dilution in 1·5 per cent. neopeptone in 1 per cent saline at which they will produce spreading of sodium deoxyribonucleate in standardised ACRA tests.

*Results.* Owing to lack of substrate, few strains of *Cl. welchii* have been examined for deoxyribonuclease production. Filtrates from 3 type-A strains contained 32-64 standard indicating doses per ml.; three type-B filtrates 8-64 doses; one filtrate from a type-C strain had no activity undiluted, but another, concentrated about 30 times, had about 16 standard indicating doses per ml. Two filtrates from type-E strains had only 4-8 doses; another, concentrated about 40 times, 1000 standard indicating doses per ml. Two type-F strains had 32 and 64 doses per ml. respectively.

*Test for* Cl. welchii *antideoxyribonuclease.* Serum GWP 2081 was chosen as standard and given an arbitrary antideoxyribonuclease value of 180 units. Tests, in general similar to those used for antihyaluronidase, were carried out at 2- or 4-unit levels; the test dose contained 15 or 30 standard indicating doses. Table VIII gives the serum values against one *Cl. welchii* type-A and one type-F filtrate. Considering the errors of the test, the agreement is fair; only one serum (EX 1263) gives discrepant values which are so far unexplained; the rest leave little doubt that the same deoxyribonuclease is present in both *Cl. welchii* type-A and type-F filtrates.

TABLE VIII

*Serum antideoxyribonuclease values against* Cl. welchii *type-A and type-F filtrates in ACRA tests (substrate sodium deoxyribonucleate)*

| Serum | Serum values against filtrate of | |
|---|---|---|
| | type A | type F |
| EX 1254 | 130, 140 | 150, 180 |
| EX 1263 | 140, 150, 140, 100, 100 | <40, <50, <30, <7 |
| EX 1274 | 490, 390, 390 | 480, 510 |
| EX 1275 | 140, 120 | 230, 200, 190 |
| RX 5326 | 50, 45 | 60, 65 |
| RX 5457 | 50, 50, 40 | 45, 45 |
| RX 5526 | 410, 420, 300, 320 | 670, 510, 540, 450 |
| RX 5527 | 180, 120, 120, 175 | 200, 215, 215 |
| GWP 160 | 35, 40 | 35, 45 |
| GWP 2081 * | 180 | 180 |

* Standard.

## *Comparison of the ACRA test with McCarty's alcohol precipitation test*

McCarty (1949) described a test for streptococcal deoxyribonuclease and antideoxyribonuclease which depends on the fact that alcohol precipitates sodium deoxyribonucleate as a fibrous mass. After digestion of the deoxyribonucleate with deoxyribonuclease, addition of alcohol produces only a light floccular precipitate.

*Test for substrate.* Dissolve sodium deoxyribonucleate to a convenient concentration in the veronal buffer used for the ACRA test and prepare from it two-fold dilutions in 0·5 ml. volumes of veronal buffer; add to each 1·5 ml. of protein hydrolysate diluted 1 in 5 in distilled water (this proved more satisfactory than neopeptone solution). Mix by inversion, incubate in a water-bath at 37° C. for one hour, remove from bath, cool rapidly in ice-cold water, add 2 ml. of 95 per cent. alcohol and mix by gentle shaking. Read the tests directly from the fibrous mass formed. If sufficient substrate is present, a firm fibrous clot will float to the surface; as the amount of substrate diminishes only *threads appear and finally only a light floccular precipitate is formed.* One batch of substrate gave a good clot at 0·13 per cent., a smaller clot at 0·065 per cent. and threads at 0·033 per cent.; we used a concentration of 0·13 per cent. in tests.

*Test for* Cl. welchii *deoxyribonuclease.* Filtrates can be tested for deoxyribonuclease by estimation of the maximum dilution in protein hydrolysate that will, under standard conditions, reduce the amount of sodium deoxyribonucleate to that which will form only threads in alcohol precipitation tests.

*Test for* Cl. welchii *antideoxyribonuclease.* GWP 2081 was used as the standard, as in ACRA tests, at a value of 180 units. Sera were tested against it at the 2-unit level at 20 per cent. differences; at this level the test dose contains 30 standard indicating doses.

To the test dose at 2 units add amounts of test sera differing by 20 per cent.; add diluted protein hydrolysate to 1·5 ml., mix by inversion and allow to stand 30 mins. Add 0·5 ml. of diluted substrate, incubate in a water-bath at 37° C. for one hour, remove, cool rapidly in ice-cold water, and add 2 ml. of 95 per cent. alcohol to each tube. Take as end-point the tube containing most antitoxin that shows threads. *Both* crude and refined sera diluted less than 1 in 20 were precipitated by alcohol under the conditions of the test; it was therefore impossible to test sera whose values were less than 40 units.

Table IX gives the average values for a number of sera tested for anti-deoxyribonuclease by the ACRA and alcohol precipitation methods; the results agree fairly well, showing that both tests estimate the same antideoxyribonuclease.

TABLE IX

*Comparison of serum values against a* Cl. welchii *type-A filtrate in ACRA and alcohol precipitation tests, using sodium deoxyribonucleate as substrate, to show that both tests measure the same antigen and antibody*

| Serum | Serum values in | |
|---|---|---|
| | ACRA tests | alcohol precipitation tests |
| EX 1254 | 130, 140 | 130 |
| EX 1263 | 140, 150, 140, 100, 100 | 110 |
| EX 1274 | 400, 300, 300 | 300 |
| EX 1275 | 140, 120 | 175 |
| RX 5326 | 50, 45 | 40 |
| RX 5457 | 50, 50, 40 | 30 |
| RX 5526 | 410, 420, 300, 320 | 550, 500 |
| RX 5527 | 180, 120, 120, 175 | 195 |
| GWP 2081 * | 180 | 180 |

* Standard.

*Nomenclature.* As table X shows, the deoxyribonuclease present in *Cl. welchii* type-A and type-F filtrate is antigenically distinct from the other known antigens of *Cl. welchii* types A and F ; we therefore propose to call it *Cl. welchii* Nu-antigen ($\nu$-antigen).

TABLE X

*Comparison of* Cl. welchii *antideoxyribonuclease values of sera with their anti-*$\alpha$*, anti-*$\beta$*, anti-*$\theta$*, anti-*$\kappa$*, and antihyaluronidase values, to show that* Cl. welchii *deoxyribonuclease is antigenically distinct*

| Serum | Serum values | | | | | |
|---|---|---|---|---|---|---|
| | Anti-$\alpha$ | Anti-$\beta$ | Anti-$\theta$ | Anti-$\kappa$ | Antihyaluronidase | Average antideoxyribonuclease value |
| EX 1254 | 5·5 | 2,800 | 6 | <20 | <5 | 135 |
| EX 1263 | 3·1 | 800 | 1·8 | <20 | <5 | 116 |
| EX 1274 | 4·25 | 6,750 | 19 | <0·5 | <0·5 | 420 |
| EX 1275 | 3·8 | 230 | 28 | <0·25 | <5 | 130 |
| RX 5326 | 1650 | 0·5 | 640 | 2500 | 145 | 47 |
| RX 5457 | 620 | 0·5 | 16·5 | 5 | 1500-2000 | 46 |
| RX 5526 | 6 | 11,000 | 3 | <20 | <1·5 | 375 |
| RX 5527 | 3·3 | 1,000 | 15·5 | <20 | <1·6 | 145 |
| GWP 160 | 4500 | 0·5 | 16 | 6900 | 750 | 47 |
| GWP 2081 * | 2700 | <0·5 | 180 | 3500 | 560 | 180 |

* Standard for antideoxyribonuclease.

## SUMMARY

The ACRA test can be modified to test with considerable accuracy for hyaluronidase and deoxyribonuclease and to calibrate sera for antihyaluronidase and antideoxyribonuclease.

By its use it can be shown that the same hyaluronidase (*Cl. welchii* $\mu$-antigen) is produced by some *Cl. welchii* type-A strains, all type-B strains and some type-D strains examined. No type-C, -E or -F strains produced any hyaluronidase ; the addition of hyaluronic acid to our medium made no difference to hyaluronidase production by any of the strains tested.

Similarly it can be shown that *Cl. welchii* types A and F produce a deoxyribonuclease (*Cl. welchii* $\nu$-antigen) and that the ACRA test and McCarty's alcohol precipitation test estimate the same antigen and antibody.

We should like to thank Dr E. Bidwell for supplies of hyaluronic acid and sodium deoxyribonucleate, Mrs I. Batty for determining the anti-$\alpha$ and anti-$\theta$ values of some of the sera, Miss J. C. Waite and Miss H. E. Ross for many bacterial filtrates, and Miss E. Hart and Miss B. Sievewright for considerable technical assistance.

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577 . 15 : 576 . 851 . 252

# STAPHYLOCOCCAL PHOSPHATASE, GLUCURONIDASE AND SULPHATASE

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BARBER *et al.* (1948) carried out experiments to determine what organisms may be responsible for the destruction of " pregnandiol-like glucuronide " (Bissot *et al.*, 1948) in unpreserved human urine. A number of organisms likely to be present in non-catheter specimens of urine, such as *Bact. coli*, *Strep. fæcalis*, and staphylococci were tested, and it was found that certain strains of *Staph. albus* destroyed both pregnandiol-like glucuronide and pregnandiol glucuronidate. Since a few strains of *Staph. pyogenes* tested at the same time were without apparent effect on these compounds, it was considered desirable to make a further study of the distribution of glucuronide-destroying enzymes among staphylococci. While studying glucuronidase production, the same organisms were tested for phosphatase and, at the suggestion of Professor L. Young, for sulphatase.

The use of phenolphthalein phosphate as a substrate in the detection of bacterial phosphatase was first recommended by Bray and King (1942). The advantage of this compound for such tests is obvious owing to the ease with which free phenolphthalein can be detected. We decided, therefore, to use phenolphthalein glucuronide, phenolphthalein phosphate and phenolphthalein sulphate as substrates for the present investigation.

## METHODS

*Substrates.* Phenolphthalein diphosphoric acid was prepared from phenolphthalein and phosphorus oxychloride in pyridine-chloroform according to the procedure of Fishman *et al.* (1948). Some of the gummy mass of phenolphthalein diphosphoric acid so obtained was dried in a vacuum desiccator over calcium chloride and used for the phosphatase tests. Phenolphthalein diphosphoric acid, unlike the calcium salt—the preparation used by Bray and King (1942), is readily soluble in water. The solution is of course acid, but can be brought to a neutral or slightly alkaline *p*H by dilution with phosphate buffer or a bacteriological medium at *p*H 7·4. A sample of the solid was converted to the sodium salt and purified according to the procedure of Huggins and Talalay (1945). The pure sodium salt was analysed for total phosphorus and for sodium. Two analyses gave values for P of 10·61 and 10·42 per cent.; the calculated P value for $C_{20}H_{13}O_{11}P_2Na_5$ was 10·24 per cent. The corresponding figures for Na were 11·9 and 11·4 per cent.; calculated value 19·0 per cent. The calculated values for the trisodium salt of the diphosphate, $C_{20}H_{15}O_{11}P_2Na_3$, were P, 11·04 per cent.; Na, 12·28 per cent. It is not possible to distinguish between the penta- and trisodium salts from the analytical figures for sodium, but it is unlikely that the compound is not a diphosphate. It will be noted that Huggins and Talalay also found anomalous figures, namely Na, 11·68 per cent.; P, 9·46 per cent.

The bulk of the phenolphthalein diphosphoric acid was made into a neutral, essentially pyridine-free solution. This was injected into rabbits at first in two simultaneous daily injections of 5 ml., each containing approximately 500 mg., and later in two simultaneous daily doses of half this amount. The rabbits (8 in all) were kept in metabolism cages and about 60 g. of phenolphthalein phosphate administered to them. Two died under the large dosage and were replaced, but all survived the lesser dosage. They were fed on cabbage and carrots, with water *ad libitum*. The urine was collected under toluene and processed according to Fishman *et al.* for phenolphthalein glucuronide. The yield of the crystalline cinchonidine salt of phenolphthalein glucuronide, twice recrystallised from hot methanol by the addition of hot ethyl acetate, was 10·1 g. An approximately equal quantity of cruder material was also obtained. The purified cinchonidine salt gave 93·5 per cent. of the theoretical quantity of phenolphthalein for a salt with one molecule of methyl alcohol of crystallisation when hydrolysed by boiling with 4*N*HCl. A nearly 0·01 *M* solution of sodium phenolphthalein glucuronidate was prepared from the crystalline cinchonidine salt by the procedure of Talalay *et al.* (1946). This was diluted as required for the glucuronidase tests.

Phenolphthalein sulphate is a new compound and was prepared by Professor L. Young.

*Preparation of medium.* Stock solutions, approximately 0·01 *M*, of the three substrates were prepared in water, sterilised by Seitz filtration and stored in a refrigerator. For tests, these solutions were added separately, with aseptic precautions, to broth consisting of Lab-Lemco 1 per cent., peptone (Evans) 1 per cent. and NaCl 0·5 per cent. at *p*H 7·4-7·6. The final concentration of the substrate used was in most cases 1/50th of the stock solution (*i.e.* 0·0002 *M*, or approximately 0·01 per cent.). Higher concentrations increased the rate of liberation of free phenolphthalein; lower concentrations gave fewer positive reactions.

It was found that the solutions were stable at a temperature of 100° C. when diluted 1 in 50 in the buffered medium at *p*H 7·4 used for the tests. The substrates were hydrolysed, however, at higher temperatures or if heated in acid solution.

*Test for enzyme production.* Broth plus substrate was inoculated with the organism to be tested and incubated at 37° C. At various intervals a sample was removed and made just alkaline by the careful addition of *N*/10 NaOH, when a pink colour develops in the presence of free phenolphthalein. (With excess of alkali the pink colour obtained with free phenolphthalein disappears.) Unless a positive result was obtained earlier, the cultures were kept in the incubator for from 7 to 14 days.

*Sources of staphylococci.* Tests were carried out on 160 coagulase-positive strains of staphylococci, hereafter referred to as *Staph. pyogenes*, and 75 coagulase-negative strains. The former were isolated from cases of staphylococcal infection or nasal carriers in seven different London hospitals, the Kent and Canterbury Hospital and St Mary's Hospital, Manchester. The date of isolation ranged from 1947 to the time of the present investigation; 80 of the strains were penicillin-sensitive and 80 penicillin-destroying. Of the coagulase-negative staphylococci, 55 were recently isolated from the anterior nares of students and staff of this hospital and 20 from pathological specimens (12 nasal swabs and 8 miscellaneous) from four London hospitals.

## RESULTS

*Phosphatase* From table I it will be seen that *Staphylococcus pyogenes* and coagulase negative staphylococci differ significantly in their production of phosphatase All the 160 strains of *Staph pyogenes*

TABLE I

*Phosphatase production by staphylococci*

| Phosphatase production | Coagulase positive | Coagulase negative |
|---|---|---|
| Positive within 24 hours * | 160 | 1 |
| Negative at 24 hours<br>Positive in 2 7 days | 0 | 54 |
| Negative at 7 days | 0 | 20 |
| Total | 160 | 75 |

* All coagulase positive strains gave this reaction in 18 hours or less the single coagulase negative strain in this category gave a reaction between 18 and 24 hours

liberated sufficient free phenolphthalein to give a deep red colour on the addition of alkali in 18 hours or less Only 1 of the 75 coagulase negative strains gave a comparable result, namely positive in 18-24 hours This strain was isolated in association with *Staph pyogenes* from the anterior nares of a member of the staff of this hospital Of the other coagulase negative strains, 54 gave a positive reaction in from 2 to 7 days and 20 were negative at the end of a week

*Glucuronidase* The number of strains liberating phenolphthalein from phenolphthalein glucuronide is given in table II It will be seen that 6 (3 7 per cent ) of 160 strains of *Staph pyogenes* and 12 (16 per cent ) of 75 coagulase negative staphylococci gave positive results The time taken ranged from 1 to 7 days Of the 6 coagulase-positive strains, 4 took from 4 to 7 days and 2 from 1 to 3 days , the time taken by the 12 coagulase negative strains was 4 7 days for 4 strains and 1-3 days for 8 strains Thus the coagulase negative strains liberated free phenolphthalein more rapidly than the coagulase-positive strains

TABLE II

*Glucuronidase production by staphylococci*

| | Coagulase positive | Coagulase negative |
|---|---|---|
| Glucuronidase positive | 6 (3 7 per cent ) | 12 (16 per cent ) |
| Glucuronidase negative | 154 | 63 |
| Total | 160 | 75 |

Of the 6 glucuronidase-producing strains of *Staph. pyogenes*, 3 were isolated from nasal swabs, 2 from ear swabs and 1 from the conjunctiva. Nine of the 12 coagulase-negative strains liberating phenolphthalein were from the anterior nares and 1 was from the air of a maternity department. The source of the remaining two is unknown.

*Sulphatase.* None of the strains of staphylococci tested was capable of liberating phenolphthalein from phenolphthalein sulphate to any considerable degree. Two strains of *Staph. pyogenes*, one isolated from the anterior nares and one from a specimen of fæces, gave a weakly positive reaction after 7-8 days' incubation. None of the coagulase-negative strains gave a positive reaction (table III).

TABLE III

*Sulphatase production by staphylococci*

| Sulphatase production | Coagulase-positive | Coagulase-negative |
|---|---|---|
| Sulphatase-positive . . . . | 2 | 0 |
| Sulphatase-negative . . . . | 158 | 73 |
| Total . . . | 160 | 73 |

In contrast to this, an aerobic sporing bacillus, encountered as a contaminant, gave a positive reaction in 5 days and a strain of *Salm. paratyphi* B in 4 days.

### *Correlation of results and relation to other findings*

*Staph. pyogenes.* Of the 2 sulphatase-positive strains, 1 also produced glucuronidase and 1 did not. As shown in table IV, there was no correlation between the production of these enzymes and of penicillinase.

TABLE IV

*Coagulase-positive staphylococci*

|  | Penicillin-sensitive | Penicillin-destroying |
|---|---|---|
| Glucuronidase-positive . . . . | 3 | 3 |
| Sulphatase-positive . . . . | 1 | 1 |

*Coagulase-negative staphylococci.* Table V relates phosphatase and glucuronidase activity. It will be seen that the two enzymes do not go together. Of the 12 glucuronidase-positive strains, 5 also produced phosphatase, 7 did not.

All coagulase-negative strains were also tested for mannitol fermentation. The results in relation to glucuronidase and phosphatase are given in table VI. Again there is no apparent correlation between any of these activities.

TABLE V

*Coagulase negative staphylococci*

| | Phosphatase positive | Phosphatase negative |
|---|---|---|
| Glucuronidase-positive | 5 | 7 |
| Glucuronidase negative | 50 | 13 |
| Total | 55 | 20 |

TABLE VI

*Coagulase negative staphylococci*

| | Mannitol positive | Mannitol negative |
|---|---|---|
| Glucuronidase positive . | 4 | 8 |
| Glucuronidase negative | 10 | 53 |
| Total | 14 | 61 |
| Phosphatase positive . | 8 | 48 |
| Phosphatase negative | 6 | 13 |
| Total | 14 | 61 |

### *Effect of concentration of substrate*

Two strains of coagulase-negative staphylococci showing glucuronidase activity were each inoculated into tubes of broth containing three different concentrations of phenolphthalein glucuronide, namely 0·0025 per cent. in tube 1, 0·01 per cent. in tube 2 (equalling the concentrations used in previous tests) and 0·1 per cent. in tube 3. After 48 hours at 37° C., the amount of free phenolphthalein was estimated The results are shown in table VII. It will be seen that the fourfold increase in substrate between tubes 1 and 2 caused a considerable increase in the amount of phenolphthalein liberated, whereas the further tenfold increase in substrate between tubes 2 and 3 had comparatively little effect on the amount of phenolphthalein liberated. A third strain, recorded as negative in table II, was found to liberate 2·3 $\mu$g./ml. of phenolphthalein when grown in broth containing 0·1 per cent. of substrate.

Four coagulase-negative strains were tested qualitatively after 24 hours' incubation in 3 different concentrations of phenolphthalein phosphate, namely 0·01, 0·05 and 0·1 per cent. Two of the strains gave a negative result in all three tubes ; these two strains had both been negative after 7 days in the previous tests. Two strains gave a positive reaction in the tube containing the highest concentration of substrate (0·1 per cent.) but were negative in the other two tubes ; these strains gave a positive result in 2 and 4 days respectively in the previous tests, when the concentration of substrate was 0·01 per cent.

TABLE VII

*Effect of concentration of substrate on amount of phenolphthalein liberated*

| Concentration of substrate (g. per 100 ml.) | Phenolphthalein liberated | | | |
|---|---|---|---|---|
| | Strain P6009 | | Strain M3994 | |
| | µg./ml. | Percentage of total present | µg./ml. | Percentage of total present |
| 0·0025 . . . . | 6·6 | 54·9 | 10·3 | 85·8 |
| 0·01 . . . . | 18·0 | 30·0 | 22·4 | 37·3 |
| 0·1 . . . . | 18·4 | 3·1 | 24·1 | 4·0 |

## DISCUSSION

In 1932 Gordon and Cooper demonstrated the presence of glycerophosphatase in chloroform-killed suspensions of *Staph. aureus* and *Bact. coli*, and in 1933 Boivin and Mesrobeanu also recorded phosphatase activity of these organisms. With acid-insoluble phosphate substrates, the latter workers found greatest activity at *p*H 10, whereas with acid-soluble phosphates the optimum *p*H was 6. They favoured the view that these organisms had two distinct enzymes but could not rule out the possibility that the results were due to a single enzyme, the optimum *p*H varying with the substrate.

The introduction by Bray and King (1942) of phenolphthalein phosphate as a substrate has made the detection of bacterial phosphatase relatively simple. These workers (Bray and King, 1943 ; King, 1943) studied a large number of micro-organisms belonging to a wide variety of species for phosphatase activity by incorporating phenolphthalein phosphate in an egg medium. The great majority of the organisms studied showed some activity, but 60 strains of *Corynebacterium diphtheriæ* all gave negative results. Ten strains each of *Staph. aureus* and *Staph. albus* (coagulase test not recorded) all gave a strongly positive reaction. The amount of substrate used by these workers was about 40 times that used in the present investigation, which may account for their finding no apparent difference in reaction between strains of *Staph. aureus* and *Staph. albus*. Bray and King (1943) found that reduction in the amount of phenolphthalein phosphate in the medium eliminated weak reactors. In the present investigation, it was found that an increase in the amount of substrate accelerated positive reactions in coagulase-negative staphylococci and the concentration used for the tests was that giving maximum differentiation.

Several studies of bacterial glucuronidase have appeared recently. From cultures of *Bact. coli*, Buehler *et al.* (1949) and Smith and Mills (1950) prepared cell-free filtrates which were able to liberate the aglycones from β-D-glucuronides. The enzyme or enzymes responsible have been loosely classified as β-glucuronidase, although no tests of their specificity or mode of action have been made. Karunairatnam and Levvy (1950) demonstrated an enzyme liberating free phenol and phenolphthalein from their respective glucuronides in cell-free preparations from medium-sized micro-organisms in the rumen of sheep. Buehler *et al* showed that β-glucuronidase production by *Bact. coli* was stimulated by the presence of menthol glucuronide in the medium. Smith and Mills (1950) found that glucuronidase was not produced by strains of *Bact. coli* grown in nutrient agar or peptone medium, but that transference to a medium containing 0·1 per cent. menthol glucuronide stimulated glucuronidase production within 24 hours. For maximal production of the enzyme these workers recommended 0·5 per cent. menthol glucuronide in the medium, although with this concentration the amount of free menthol liberated was sufficient to inhibit growth completely. It is possible, therefore, that in the present investigation more strains of staphylococci would have given positive results if a greater concentration of substrate had been used.

## SUMMARY

We tested 160 strains of *Staph. pyogenes* and 75 strains of coagulase-negative staphylococci for their capacity to liberate free phenolphthalein from phenolphthalein phosphate, phenolphthalein glucuronide and phenolphthalein sulphate in fluid medium.

All 160 strains of *Staph. pyogenes* but only 1 coagulase-negative strain liberated free phenolphthalein from phenolphthalein phosphate in 24 hours or less; of the remaining coagulase-negative strains 54 gave a positive reaction with this substrate in 2-7 days and 20 were negative at the end of a week.

With phenolphthalein glucuronide 6 (3·7 per cent.) strains of *Staph. pyogenes* and 12 (16 per cent.) coagulase-negative strains gave a positive reaction.

Only 2 strains of *Staph. pyogenes* and none of the coagulase-negative staphylococci showed any sulphatase activity.

Our thanks are due to Professor L. Young for the supply of phenolphthalein sulphate and for much valuable advice and criticism.

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578 . 8 . 078 . 39 : 576 . 851 . 252 (*Staph. pyogenes*)

# IDENTIFICATION OF *STAPHYLOCOCCUS PYOGENES* BY THE PHOSPHATASE REACTION

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(Plate III)

In the previous investigation (Barber *et al.*, 1951) phenolphthalein phosphate, as recommended by King (1943), was used for the detection of phosphatase production by staphylococci. It was found that there was a close correlation between coagulase production and phosphatase production. Thus coagulase-positive staphylococci, hereafter referred to as *Staph. pyogenes*, produced sufficient phosphatase to split phenolphthalein phosphate incorporated in nutrient broth in a concentration of 0·01 per cent. in 12-18 hours, whereas coagulase-negative strains liberated phenolphthalein under these conditions either more slowly or not at all. The present investigation was undertaken, therefore, to see whether phosphatase production could be used for the detection, of pathogenic staphylococci in young cultures.

## Methods

*Phenolphthalein diphosphate agar.* A stock solution, approximately 0 01 *M*, was prepared by dissolving 0 5 g. phenolphthalein diphosphoric acid in distilled water, sterilised by Seitz filtration and stored in a refrigerator. Such a solution has a *p*H of about 2 0 and is therefore easily hydrolysed. It becomes relatively stable, however, when diluted 1 in 5 or more in a buffered medium at *p*H 7·4.

A similar solution of the sodium salt of phenolphthalein phosphate gave equally satisfactory results, and as this is a more stable compound it is probably more suitable for routine use The calcium salt, however, can not be dissolved in the required concentration.

For the preparation of medium a suitable quantity of nutrient agar at *p*H 7 4 was melted and, using aseptic precautions, 1 part of phenolphthalein diphosphoric acid solution was added to 49 parts of melted agar, giving a final concentration of 0 01 g. phenolphthalein diphosphoric acid per 100 ml. nutrient agar. The mixture was poured into Petri dishes, allowed to set and then dried in the incubator. Phenolphthalein phosphate agar thus prepared could be kept and melted for plates or slopes as required. In the tests here described, the nutrient agar consisted of 1 per cent. Lab Lemco, 1 per cent. peptone, 0 5 per cent. NaCl and 1·5 per cent. shred agar. A digest medium was shown, however, to give similar results. The concentration of substrate is important, since, within certain limits, the rate of liberation of free phenolphthalein varies directly with it. Thus with a tenfold increase in substrate a number of coagulase-negative staphylococci give positive reactions in 18 24 hours

*Collection and testing of specimens* Nasal swabs were taken with broth-moistened swabs from the anterior nares of 100 adults, 37 patients, 36 medical students and 27 laboratory staff. The swabs were plated directly on phenolphthalein diphosphate agar plates in the usual way. After overnight (16-18 hours') incubation at 37° C., the growth was held over an open bottle of ammonia, when colonies producing sufficient phosphatase to liberate free phenolphthalein become bright pink, whereas others remain unchanged. Representative colonies were selected for further investigation soon after exposure to ammonia, as the pink colour fades after some minutes.

Coagulase tests on colonies selected from all plates were carried out by the tube method of Fisk (1940). In a few instances the slide method of Cadness-Graves *et al.* (1943) was also used and gave similar results.

## RESULTS

When the phenolphthalein phosphate plates were first examined after overnight incubation, staphylococcal pigmentation had not fully developed and only a few of the plates showed typical *aureus* colonies. On exposure to ammonia, however, 42 of the 100 plates showed phosphatase-positive staphylococci. On a few plates there were colonies of coliform bacilli which appeared pink after exposure to ammonia, but these were readily distinguished from staphylococcal colonies. Figs. 1 and 2 show typical plates. Fig. 1 is a Petri dish inoculated on the upper half with a pure growth of *Staphylococcus pyogenes* and on the lower with a pure growth of a coagulase-negative staphylococcus. Fig. 2 shows a mixture of coagulase-positive and coagulase-negative staphylococci, the former appearing bright pink owing to phosphatase production.

From 1 to 4 phosphatase-negative staphylococcal colonies from all plates and 2 phosphatase-positive staphylococcal colonies from each of the 42 plates, where these were present, were inoculated individually into tubes of Lab-Lemco broth. The selection of colonies was sometimes difficult where there was a heavy growth of phosphatase-positive staphylococci, as free phenolphthalein was then present in the surrounding medium. This gave rise to a generalised pink background, thus causing phosphatase-negative colonies in some cases to appear slightly pink. After 6-24 hours at 37° C. the broth cultures were tested for coagulase production by the method of Fisk, and in most cases retested for phosphatase.

The staphylococcal colonies picked from the 58 plates showing no phosphatase-producing colonies were in all cases coagulase-negative. Thus with these cultures there was complete correlation between coagulase production and phosphatase reaction.

From the 42 plates showing phosphatase-positive staphylococci 160 colonies were selected for the coagulase test; 85 were phosphatase-positive and 75 were negative or doubtful. Of the 85 phosphatase-positive colonies, all but 1 were coagulase-positive. The one coagulase-negative culture was retested for phosphatase and again gave a positive reaction after 18 hours incubation. Of the 75 negative or doubtful colonies, 70 were coagulase-negative and 5 coagulase-positive; the latter 5 colonies came from plates showing a heavy growth, making

30302

IDENTIFICATION OF *STAPH. PYOGENES*

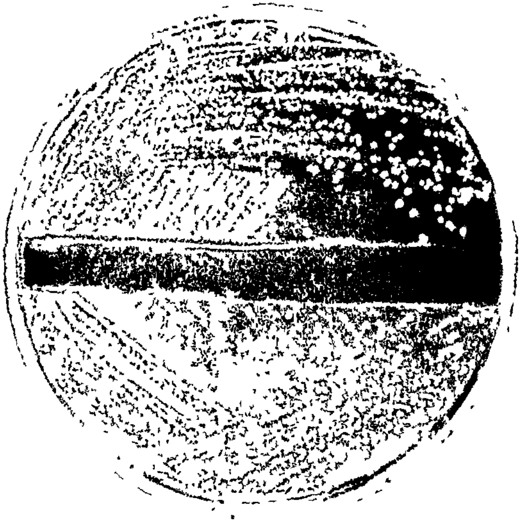

FIG. 1.—Upper half of plate; pure growth of *Staph. pyogenes* (pink): lower half; pure growth of coagulase-negative staphylococci.

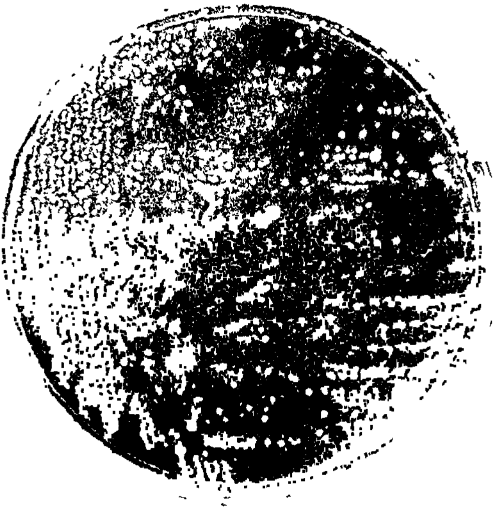

FIG. 2.—Culture as from nasal swab showing a small number of pink colonies of *Staph. pyogenes* and a heavy growth of coagulase negative (white) staphylococci.

Cultures of staphylococci grown on nutrient agar plus 0 1 per cent. phenolphthalein diphosphate for 18 hours and then exposed to ammonia. Phosphatase-positive colonies pink, phosphatase negative colonies white.

cases retested for phosphatase. All but 1 of the phosphatase-positive colonies tested were coagulase-positive. All persistently phosphatase-negative colonies were coagulase-negative.

A few colonies picked from crowded plates gave doubtful phosphatase reactions and were coagulase-positive but, on retesting, all such colonies gave a positive phosphatase reaction in 18 hours or less.

Our thanks are due to Mr A. L. Wooding for the colour photographs.

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# TWO CASES OF GIANT-CELL GRANULOMA OF THE PITUITARY GLAND

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(Plates IV-IX)

In 1917 Simmonds discovered four cases of a peculiar and unexpected granulomatous lesion of the pars anterior of the pituitary gland in a series of 2000 post-mortems. The glands, of normal size and naked-eye appearance, were patchily infiltrated with collections of lymphocytes and foci of epithelioid cells and multinucleated giant-cells of Langhans' type. No acid-fast bacilli were demonstrated. The giant-cells were numerous and sometimes appeared singly, lying amid healthy parenchymatous cells. A few foci showed fibrous regressive changes. The ages and causes of death of these patients were 63 (strangulated hernia), 73 (arteriosclerosis and cerebral softening), 61 (myocardial degeneration and cerebral softening) and 50 (myelitis and pyelonephritis). All were females whose organs showed no evidence of active syphilis or tuberculosis. In discussing the ætiology, Simmonds ruled out syphilis because of the absence of acquired or congenital specific lesions elsewhere, the absence of endarteritis in the pituitary and of inflammatory cells around the isolated giant-cells, and the atypical histology. He found it more difficult to dismiss tuberculosis and admitted that the absence of acid-fast bacilli was not necessarily significant. Although some of the cellular collections closely resembled miliary tubercles he thought most of the picture so atypical as definitely to rule out tuberculosis. Some of the giant-cells appeared to have arisen directly from pituitary parenchymatous cells. He concluded that his cases were examples of a disease *sui generis*, possibly resulting from the chronic irritation of altered pituitary secretions comparable with the analogous changes sometimes seen in the thyroid gland in elderly females.

In an exhaustive review of the literature on destructive lesions of the pituitary found *post mortem*, Sheehan and Summers (1949) grouped 18 cases, including Simmonds's four cases, under the heading of "giant cell granuloma". These case reports form a remarkable collection. Many of them are difficult to interpret and we do not propose to analyse them here. We agree with Sheehan and Summers's summing up (p. 324) that "The group may include lesions due to several different causes, such as syphilis, tuberculosis, or sarcoidosis, but most of the cases appear to be examples of a specific disease process whose ætiology is unknown". Oelbaum and Wainwright (1950) have recently described a further case and have come to similar conclusions.

Sheehan and Summers also separated from a very large series of chronic fibroid lesions five severe and four moderately severe cases of pituitary scarring which they considered to be examples of healed granuloma of the anterior lobe. Two of the moderate lesions were their own cases and are described in detail.

Each of the present authors chanced upon an example of giant-cell granuloma of the pituitary in the subacute stage, and, having independently consulted Professor Sheehan, were encouraged by him to place them on record. Though our findings probably further obscure the ætiology, we are impressed by their morphological identity with Simmonds's original four cases.

## Case I

### *Clinical history*

Mrs A. C. first came to Hammersmith Hospital in 1936 at the age of 40, complaining of tiredness for two years and attacks of pain in the chest for two weeks. She had worked at tailoring from age 14, married at 26 and had had two normal pregnancies at 27 and 30 years of age. Her periods, which started at 14, were irregular, and often profuse. At 30 she complained of swelling at the root of the neck, with cough and huskiness, and was recommended to take iodine in milk and to rest for 8 weeks. The symptoms resolved and her general health remained good for the next eight years, when at the age of 38 her menstrual periods ceased abruptly and she began to feel increasingly weak, tired and shaky, and suffered from headaches. During the previous two weeks she had had attacks of pain in the upper left chest lasting 10 minutes and associated with difficulty in breathing and a desire to take a deep breath. On examination she appeared moderately well-nourished, weighing 8 st. 1 lb., and fairly calm; pulse 80, blood pressure 130/85. The thyroid gland was moderately enlarged, firm and nodular. Urine :—No albumin, no sugar. Blood count :—Hb. 12·9 g. per 100 c.c., R.B.C. 5,300,000, W.B.C. 4000, polymorphs 1200, lymphocytes 2720, large mononuclears 80 per c.mm. Chest X-ray :—Trachea not compressed, calcified node in right upper zone, no definite evidence of substernal thyroid. Gynæcological examination :—Senile post-menopausal uterus. Her symptoms were considered to be due chiefly to home worries; she was reassured and given a bromide mixture.

She came to hospital again two years later, in 1938, complaining of "very bad nerves" and loss of weight. Examination showed weight 7 st. 9 lb., but otherwise no change in her condition. She was sent on to a psychiatrist who considered that she was suffering from a chronic mild depressive condition, that the sudden menopause had acted both physically and psychologically in increasing her anxiety and depression and that the uterine changes and general appearance suggested premature ageing, both mental and physical.

She was finally admitted to Hammersmith Hospital in 1939 in coma, having been found unconscious in bed at 6 a.m. that morning. She had been feeling off colour the day before. Her arms were in rigid flexion and she made occasional jerky movements of the wrists and fingers The legs were in rigid extension Arm, leg and abdominal reflexes were absent, plantar responses were extensor The urine contained acetone but no sugar Blood pressure 130 systolic, diastolic undeterminable Lumbar puncture produced clear fluid at 90 mm pressure, rising to 160+ on jugular compression and dropping to 50 mm after withdrawal of 10 c c The generalised rigidity gave way to flaccidity and she died 5½ hours after admission, having been in coma for not less than 8 nor more than 16 hours

## *Post mortem examination*

This was done 3 days after death by the late Dr T H Belt, who reported as follows

A pale, thin woman with rather sunken eyes, weighing 6 st 9 lb Height 5 ft 4 in

The *brain* had been hardened *in situ* by formol injection the day before the post mortem The dura was rather tense and the convolutions were flattened, but the hemispheres were symmetrical and there was no evidence of hæmorrhage The brain stem was soft and tore on slight manipulation Section across the mid-brain showed softening in the region of the substantia nigra on both sides and in the cerebral peduncles The *cerebral arteries* were not notably thickened and no occlusion could be made out The *pituitary* was of normal size, the *thyroid* nodular and moderately enlarged The appearance on section was that of multiple fœtal adenomata with calcified centres, one of these was of walnut size The *abdominal aorta* showed slight roughening around the mouths of the intercostal vessels, with deposits of pale pink staining (? platelet) material which could be scraped away The *lungs* had a slight meaty quality in the lower lobes but no definite pneumonia or pleurisy *Gastro intestinal tract* showed no notable change *Liver* (1210 g ) pink and friable *Spleen* (200 g ) capsule slightly adherent to diaphragm and showing button like plaques of whitish thickening, pulp fairly firm, corpuscles inconspicuous The *adrenals* were small and thin, the cortex being only about 1 mm thick The *heart* (290 g ), *kidneys*, *ureters*, *pancreas*, *bladder*, *uterus* and *vertebræ* showed nothing remarkable *Ovaries* small, white and tough

## *Histology*

There were available for study at this date paraffin blocks and slides of various organs and a limited number of stained sections, including frozen sections stained for fat, but no paraffin blocks, of the pituitary or adrenals

*Pituitary*, of normal size, shows on horizontal section a crescentic pars anterior abutting on the pars nervosa, but enclosed in a thickened nodular capsule There is complete disorganisation of the pars anterior (fig 1), whose normal alveolar pattern is replaced by loosely arranged degenerate pituitary cells, numerous macrophages and widely distributed multinucleated giant cells, scattered clumps and tubules of small acidophil pituitary cells, scattered eosinophil and neutrophil polymorphs, lymphocytes and occasional plasma cells, and a few collections of lymphocytes (fig. 8). The vessels are normal. The tubules are small and fœtal-like; acidophils are distributed mainly in the lateral masses. The cells which make up the background of the whole of the pars anterior vary in appearance. Some apparent lymphocytes show, in Mallory-stained preparations, gradations to recognisable chromophobes and small acidophils. There are also occasional normal-sized acidophils. The majority of the cells are finely granular, finely vacuolated mononuclears, many of whose nuclei are of the elongated twisted vesicular type seen in epithelioid cells (fig. 2). They show a fine granularity with Mallory's stain, sometimes acidophil, mostly basophil. They show every transition to giant-cells containing two or more nuclei and are considered to be mostly macrophages. The giant-cells are striking (figs. 3-5). They vary in size from about 20 to 100 $\mu$ in diameter and show from 2 to over 60 nuclei in section. Their nuclei, mostly vesicular, are peripherally distributed but some may form a central as well as a peripheral ring (fig. 4). Most of the giant-cells have finely granular cytoplasm, sometimes acidophil, mostly basophil. Some contain cytoplasmic inclusions, which are of two types, namely fragments of yellowish, brilliantly anisotropic material (fig. 5), and flaky hæmatoxyphil isotropic material resembling inspissated colloid and contained within large vacuoles (fig. 3). Frozen sections show a little fine fatty material, both in the macrophages and in the giant-cells. Though the macrophages are in general distributed haphazard, they show a tendency to be arranged in large follicles in the periphery of the pars anterior (fig. 6). The capsule of the pituitary contains definite circumscribed follicles of epithelioid-like cells both with and without giant-cells. Some of the capsular follicles are oval rather than round and are markedly hyalinised. Silver impregnation shows an abnormally broken-up reticular pattern in the pars anterior (fig. 7), with a slight increase in number of fibrils, which are thickened and collagenised in the periphery of the gland. The lymphocytic collections lie in the median part of the pars anterior (fig. 8). The eosinophil leucocytes, mostly bilobed, are scattered in moderate numbers throughout. The general structure of the pars nervosa appears normal but it contains a few small paravascular collections of macrophages, one of them associated with a giant-cell. There is quite a large pericapsular collection of macrophages and giant-cells over the pars nervosa, possibly replacing remnants of the pars tuberalis. The pars intermedia cannot be identified (fig. 8) and there are no cysts. The site of the pars intermedia is occupied by macrophages, giant-cells and chromophobes continuous with the anterior lobe lesion. No acid-fast bacilli are seen.

*Adrenals.* The cortex is reduced to a travesty, being about one-fifth of the normal width and consisting of small alveoli and strands of cortical cells rich in lipoid; pigmented cells of the zona reticularis are absent (fig. 9). This zone is separated by condensed reticulin

GIANT-CELL GRANULOMA OF PITUITARY

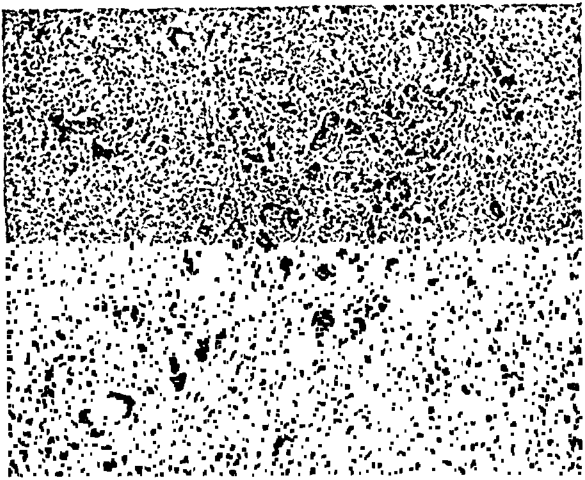

FIG. 1.—Pars anterior of pituitary of case 1. Tubules and trabeculæ of darkly staining small fœtal-like pituitary cells lie in a disorganised background of macrophages, lymphocytes and multinucleated giant-cells. Hæmalum and eosin. ×90.

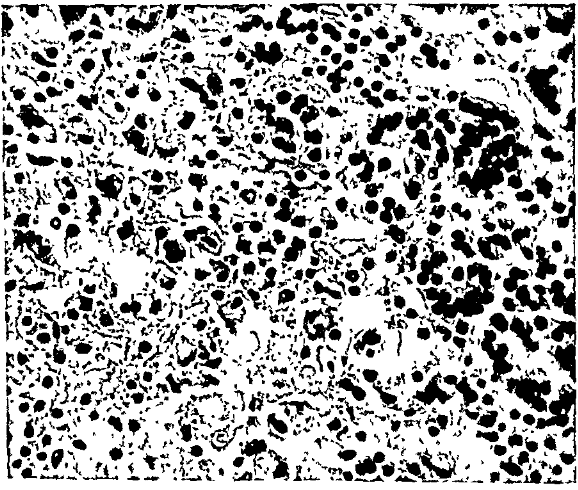

FIG. 2.—High-power view of fig. 1. The background cells are mononuclears with abundant cytoplasm, showing apparent transitions between pituitary cells and macrophages. ×380.

## GIANT CELL GRANULOMA OF PITUITARY

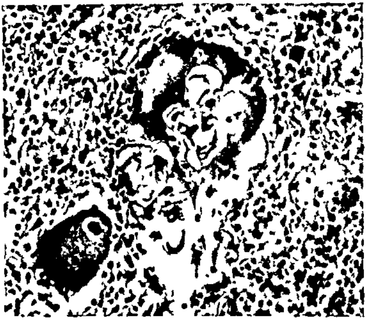

Fig 3.—Pars anterior of case I Foreign body multinucleated giant cell containing vacuoles and flaky hæmatoxyphilic inclusions H. and E ×180

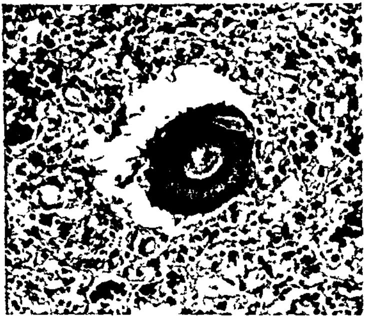

Fig 4.—Granular foreign-body giant cell showing central and peripheral rings of nuclei ×180

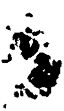

Fig 5.—Multinucleated giant-cell containing *anisotropic* inclusions, photographed *through crossed Nicol* prisms ×400.

GIANT-CELL GRANULOMA OF PITUITARY

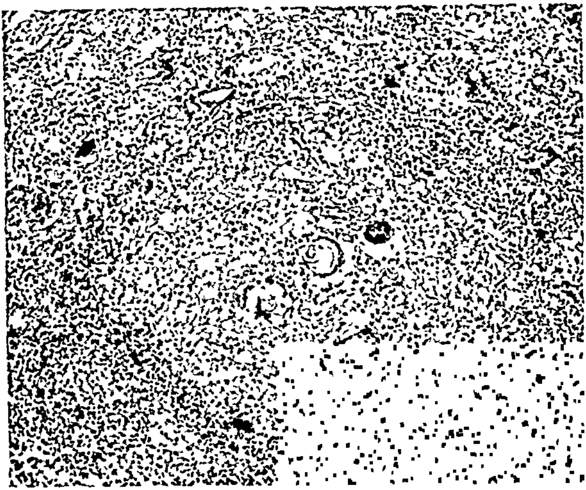

FIG. 6.—Pars anterior of case I. Peripherally situated follicle of apparent macrophages and giant cells, the latter containing inclusion bodies and vacuoles. H. and E. ×100.

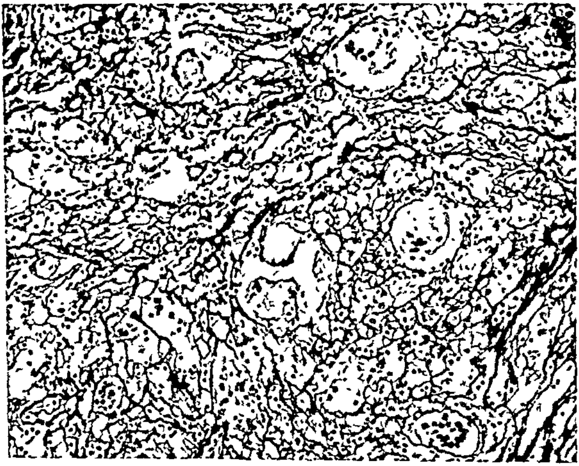

FIG. 7.—*Pars anterior* of case 1. Reticulin preparation. Granulomatous area rich in giant cells showing an irregular increase in reticulin fibres but none of the follicular arrangement typical of tuberculosis, syphilis or sarcoid. Gordon and Sweet's reticulin stain. ×180

GIANT-CELL GRANULOMA OF PITUITARY

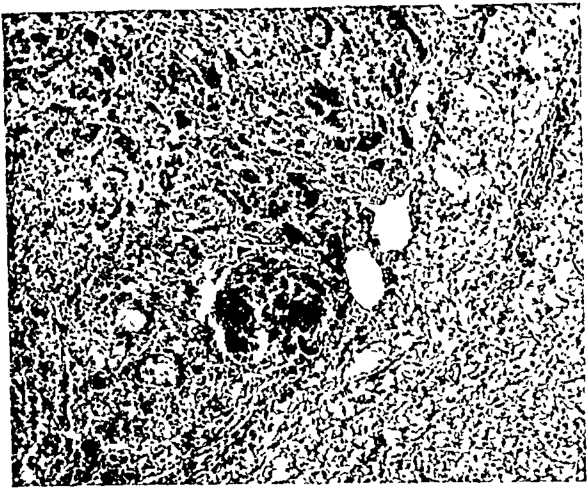

FIG. 8.—Junction of pars anterior with pars nervosa of case 1. There is no recognisable pars intermedia. The pars nervosa appears normal. The pars anterior contains a focal collection of lymphocytes and is mostly replaced by lymphocytes, macrophages and giant-cells. H. and E. ×90.

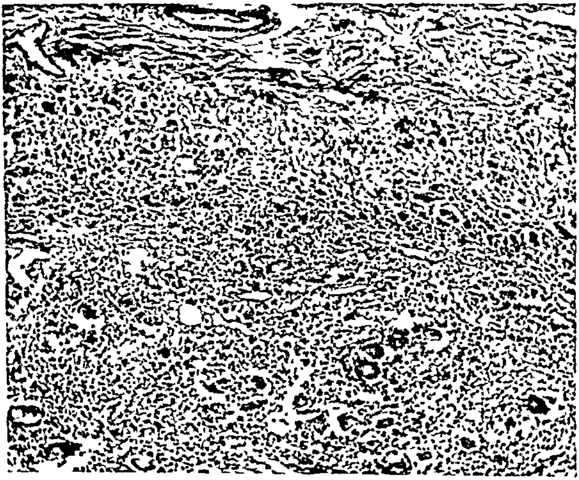

FIG. 9—Adrenal of case 1. From above downwards:—thickened capsule, surviving rim of cortex, a fine fibrous zone infiltrated with lymphocytes and finally massive replacement of adrenal structure by lymphocytes and numerous multinucleated giant-cells. H. and E. ×180.

from an expanded spongy "medulla" traversed by a normal central vein and its radicles. There are no recognisable medullary cells but some of the veins are cuffed by a narrow rim of lipoid-containing cortical cells. The medulla is expanded and replaced by macrophages, multinucleated giant-cells and lymphocytes (fig. 9). The macrophages and giant-cells are sometimes arranged in follicles, which are also present scattered in the cortex, capsule and peri-capsular tissue. The lymphocytes lie both diffusely scattered and in small collections. The picture is almost identical with that seen in the pituitary; some giant-cells contain anisotropic inclusions, but the flaky hæmatoxyphil inclusions are not seen. Reticulin fibres in the medulla are increased and in parts collagenised. No acid-fast bacilli are seen.

*Thyroid.* The gland is broken up into "adenomata" of varying morphology, between which lie small and medium-sized normal thyroid follicles lined by cuboidal epithelium, some rich, others poor in colloid. They appear moderately active rather than resting or atrophic. The adenomata are mostly made up of large colloid-filled follicles lined by cuboidal epithelium. Some of them are partially fibrosed and calcified, some show areas of a microfollicular pattern poor in colloid. There is moderate interstitial fibrosis but no lymphocytic infiltration.

*Ovary.* This is fibrotic and rich in ancient fibrosed and hyalinised corpora lutea. The peripheral stroma still contains a fair number of primordial follicles, none of which, however, shows any sign of ripening.

*Uterus.* The myometrium is atrophic and fibrous, the endometrium thin and made up of slightly dilated glands lined by non-active cuboidal and low-columnar epithelium, lying in a stroma of small, densely packed hyperchromatic spindle cells.

*Breast.* This is made up of coarse fibrous tissue traversed by a reduced number of duct systems which consist of narrow branching tubules lined by flattened epithelium and contain eosinophilic colloid material. The picture is one of partial mammary atrophy and fibrosis.

*Pancreas* (2 blocks). Islets are prominent, both in size and in number. *Liver* shows a slight excess of fibrous tissue and lymphocytic infiltration in the portal tracts. *Heart* and *kidneys* show no obvious pathological changes. *Mediastinal lymph-nodes* show intense congestion and moderate sinus reticulum-cell hyperplasia.

*Spleen.* The pulp contains one miliary nodule of fused epithelioid cells embedded in a dense reticulin network clustered around central hyalinised collagen, a lesion indistinguishable from a healing miliary tubercle. No acid-fast bacilli are present and no giant-cells.

*Lungs.* Sections show congestion and œdema, with foci of early hæmorrhagic bronchopneumonia. The terminal bronchioles and alveoli in the affected areas are filled with moderate numbers of polymorphs, fibrin and masses of red cells.

*Aorta* (2 blocks). The intima shows patchy thickening by proliferated mucoid connective tissue, the significance of which is not understood. The media is normal.

*Brain* (5 blocks) and *upper cervical spinal cord.* No gross pathological changes seen; no evidence of softening.

### *Discussion of case* 1

The above post-mortem findings throw no direct light upon the cause of the terminal coma. Cerebral thrombosis is ruled out in view of the normal non-occluded arteries in the brain and normal brain sections. An easily missed lesion of a small branch artery would have been unlikely to precipitate a deep and fatal coma. The gross disorganisation of the pituitary and adrenals points to an attack of hypoglycæmia as a possible basis for the coma, the attack perhaps brought on by a respiratory infection on the previous day, when the patient was "off colour." The bronchopneumonic lesion favours this hypothesis but does not prove it, since a deep coma of 16 hours might conceivably have provided sufficient time for the lung lesion to develop. The earlier phases of the coma were missed; we do not know if it was ushered in with convulsions. The rigidity, the extensor plantar responses and the fact that the diastolic blood pressure was unobtainable on admission when the patient was *in extremis* favour a hypoglycæmic origin and accord well with Sheehan and Summers's description.

The patient's symptoms fall into two categories, one referable to the thyroid enlargement, the other to the pituitary and adrenal failure. The symptoms of huskiness and cough at age 30 and of attacks of pain and difficulty in breathing at age 40, together with the clinical presence of a nodular goitre, correlate well with the post-mortem finding of an adenomatous thyroid with one walnut-sized nodule projecting downwards from the left lobe. The gland would appear to have shrunk somewhat during the 2 years before death. The symptoms described were probably due to pressure and there was no evidence of thyrotoxicity. The abrupt menopause at 38 followed by asthenia, shakiness and headaches, points to the pituitary lesion being of at least 5 years' standing. The premature ageing and the senile atrophied uterus fill in the hypopituitary picture. The presence of a number of surviving unripe follicles in the ovary further confirms the hypopituitary origin and prematurity of the menopause, since the ovary 5 years after a spontaneous menopause should be bereft of ova. The adrenal cortical atrophy found at autopsy is typical of long-standing hypopituitarism, the degree of which, however, is not easy to assess. The sections available show an 80-90 per cent. loss of cells of the pars anterior. The great uterine atrophy points to total loss of pituitary gonadotropic function, while the degree of thyroid survival suggests a non-total loss of pituitary function. The progressive loss of weight from 8 st. 1 lb. in 1936 to 6 st. 9 lb. in 1939 favours a progressive loss of pituitary function during that time.

The interpretation of the finding of a possible scarred miliary

GIANT-CELL GRANULOMA OF PITUITARY

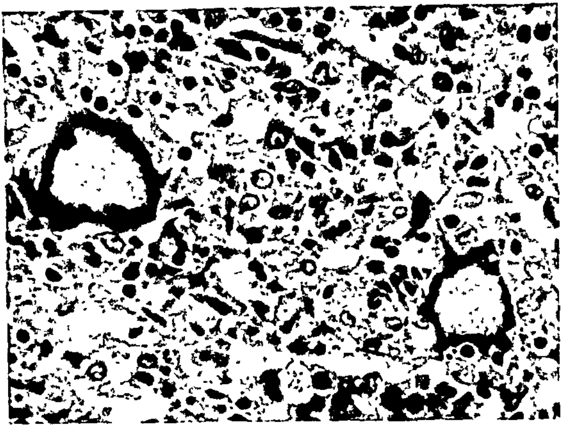

FIG. 10.—Pars anterior of pituitary of case 2. Two multinucleated giant-cells are lying in diffuse granulation tissue containing a few pituitary cells. H. and E. ×600.

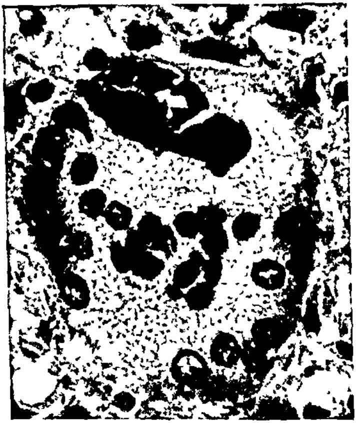

FIG. 11.—Pars anterior of pituitary of case 2. A large multinucleated foreign-body giant-cell with a large hæmatoxyphilic inclusion occupies most of the field. At one end of the inclusion there is a transparent, laminated yellowish brown structure. H. and E. ×900.

FIG. 12.—The same as fig. 11, photographed through partially crossed Nicol prisms. The anisotropic material corresponds to the laminated transparent structure seen in fig. 11.

tubercle in the spleen is difficult. One cannot gauge its significance in relation to the pituitary and adrenal lesions, which were histologically almost certainly non-tuberculous. The splenic tubercle may be simply evidence that the patient was debilitated and had suffered a recent lighting up of an old tuberculous infection—a not unusual post-mortem finding in the lungs and mediastinal lymph-nodes. The nature of the giant-cell granuloma is discussed later in conjunction with the findings in case 2.

## Case II

### *Clinical history*

Mr P. K., aged 60 years, a retired policeman, was admitted to Guy's Hospital on 17th July 1949 for increasing breathlessness on exertion and increasing lassitude during the past year. From time to time he had had pains in the arms, shoulders, lumbar regions and right thigh and knee. He said he had lost about 3 stones in weight in the last two or three years and his appetite was poor.

Previous complaints included diphtheria at age 32, scarlet fever at age 46 and, in recent years, a winter bronchitis for which he had been discharged from the police force at age 58. He had served in France in the 1914-18 war and in the Port of London Authority Police from 1919 to 1947. There was no relevant family history.

During the nine months before admission he had attended Guy's Hospital out-patient department on several occasions, when he was found to have hypertension (about 180/130) and occasional slight œdema of the ankles. His E.C.G. showed a left bundle branch block, his chest X-ray a hypertrophied left ventricle with prominent aorta. The erythrocyte sedimentation rate was 26 mm. in the first hour; hæmoglobin 70 per cent.

Shortly before admission he became breathless while at rest and his heart began fibrillating. On admission his temperature was 96·5° F., pulse 90 and respirations 28 per minute. There was slight œdema of the ankles and the heart sounds were faint and irregular. No veins were visible in the neck when he sat up. B.P. 155/95. In the lungs there were moist sounds at both bases posteriorly but no dullness. There was no finger clubbing. The liver was four fingers' breadth below the costal margin. Movement of the right hip was limited in all directions by pain and there were crepitations in the right knee.

He improved with rest in bed and digoxin. The pulse fell to 60, the B.P. to 130/85, the liver became only just palpable and the ankle œdema disappeared. On 26th July 1949, while washing, he suddenly collapsed and died immediately.

### *Post-mortem examination*

A well-built obese subject with slight œdema of the ankles.

The *pericardial sac* contained a little blood-tinged fluid. The *heart* (500 g.) was large and globular and the left ventricle hypertrophied. There were signs of old scarring in the muscle. All valves showed slight atheroma. The *left coronary artery* was at least three-quarters occluded proximally by old calcified and laminated atheroma, while its anterior descending branch was completely blocked by recent thrombus. The *right coronary artery* was at least half occluded by calcified atheroma. The *aorta* was markedly atheromatous, particularly the distal part of its abdominal portion.

The main *bronchi* contained an excess of tenacious mucus. Both *lungs* (r. 640 g., l. 510 g.) showed marked congestion and slight œdema.

The *stomach* contained a small quantity of undigested food. The *mesenteries* and *peritoneum* were heavily loaded with fat. The *liver* (1890 g.) was large but otherwise normal. *Bone marrow* of sternum normal. Both *kidneys* (r. 130 g., l. 150 g.) were congested and the cut surfaces were more granular than normal. There were a few petechial hæmorrhages in the pelvic mucosa.

*Adrenals, thyroid* and *pituitary gland* appeared normal.

*Brain* 1410 g. The basilar artery and the arteries of the circle of Willis showed marked atheroma but there were no abnormalities of brain or meninges.

## *Histology*

*Pituitary.* On vertical sagittal section, the gland is of approximately normal size and shape. The capsule is thickened by old fibrous tissue and in several places infiltrated with lymphocytes: in one section it contains granulation tissue. The pars anterior occupies approximately 7/8ths of the section. Its normal structure is everywhere largely replaced by granulation tissue elements (fig. 10)—lymphocytes, macrophages, giant-cells, fibroblasts (young and old), fibrous tissue and a few eosinophil leucocytes. These elements intermingle with and separate the proper cells of the pituitary—chromophobe, eosinophil and basophil cells—approximately 30 per cent. of which remain. Lymphocytes are most numerous. They are either diffusely scattered or aggregated in dense, roughly circular collections. Macrophages are similarly distributed but in smaller numbers. Giant-cells occur singly and in considerable numbers. All are of Langhans' type and contain from 6 to 27 peripherally situated nuclei, comparable in size with those of the macrophage cells, or much smaller, pyknotic and flattened. Their cytoplasm is pale, finely granular and eosinophilic, but more darkly staining around the nuclei. The cytoplasm of some of the giant-cells contains a yellowish substance which is doubly refractile (figs. 11 and 12), and in one there is a laminated, deeply blue-black calcium-like concretion (fig. 11). Occasionally it is difficult to distinguish a giant-cell from an alveolus (Erdheim gland). Usually these glands are distinguished by the homogeneous nature, even under ×950, of their central colloid and well-defined cell walls. Even so, a doubt sometimes remains that a giant-cell structure may have been a degenerate Erdheim gland.

The pars nervosa contains several collections of lymphocytes, with a few macrophages but no giant-cells. The pars intermedia cannot be made out and there are no colloid cysts visible. The stalk possesses twelve large thin-walled blood vessels, mainly on the surface. One small collection of lymphocytes is seen. There is also a small thick-walled arteriole 30 $\mu$ in diameter on the surface of the stalk, surrounded

GIANT-CELL GRANULOMA OF PITUITARY

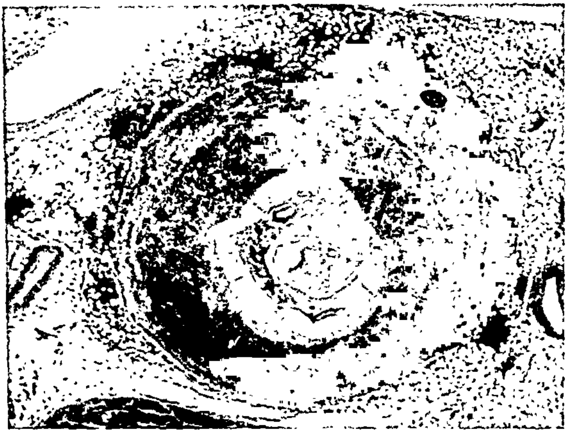

FIG. 13.—Anterior descending branch of the left coronary artery of case 2. The lumen is occluded and the media replaced by highly cellular granulation tissue many times the thickness of the normal structure. H. and E. ×15.

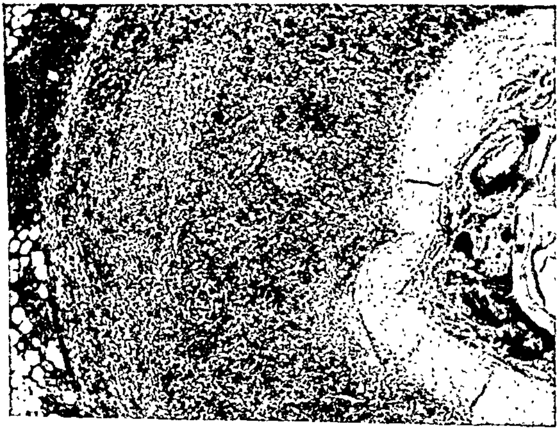

FIG. 14.—A higher-power view of the artery shown in fig. 13. H. and E. ×40.

by a circular area of granulation tissue 130 $\mu$ in diameter. This consists mainly of macrophages and (fewer) lymphocytes. The media and intima of the artery are normal. Where the stalk joins the body of the pituitary there are several small nests of epidermoid cells.

*Coronary arteries.* The lumen of a large branch of the descending branch of the left coronary is occluded by recent clot laid down on an older clot which shows some organisation (figs. 13 and 14). External to this there is an irregular band of atheroma containing lipoid spaces and calcified foci—average thickness 0·5 mm. Outside this again, instead of media, there is a band of granulation tissue—average thickness 2 mm., composed mainly of macrophage cells, fibroblasts and eosinophils, with a few lymphocytes, plasma cells and neutrophil polymorphs. Elastic staining shows that the internal elastic lamella is grossly fragmented. The external elastic lamella is represented only by occasional fragments of elastica. Outside this there is a rather indefinite layer of fibroblasts and collagen fibres surrounded by more granulation tissue but with a predominance of lymphocytes. This inflammatory process spreads into the adjacent adipose tissue (figs. 11 and 12). A small portion of attached heart muscle is largely necrotic. The right coronary artery shows similar changes but part of the vessel wall has broken down and the space is filled with recent blood clot.

*Liver.* The sinusoids are congested and there is a slight increase in the number of periportal lymphocytes. There are a few poorly formed giant-cells with 5-11 peripheral nuclei in the portal tracts.

*Spleen.* This organ is congested and there is a severe degree of rather irregular hyalinisation of its arterioles.

*Adrenals.* An artery external to an adrenal shows recent thrombosis and three others an eccentric narrowing by eosinophilic material, which also replaces the media. This does not stain for amyloid. The gland itself is congested.

*Kidneys.* These are much congested. A few glomeruli are atrophic and fibrosed. Several of the larger arteries show moderate concentric intimal thickening.

**Conclusion.** The cause of death in this case was coronary thrombosis. During life there were no definite signs or symptoms of hypopituitarism. Symptoms such as tiredness and loss of appetite could be related to the increasing heart failure.

## DISCUSSION

Deductions as to the nature of the pituitary lesions in these two cases vary according to whether we analyse the pituitary findings alone, or try to fit them in with the additional findings in the adrenals and spleen in case 1 and in the coronary arteries and liver in case 2. Sheehan and Summers were struck by the appearance of a tempting series of steps between the granuloma cells and scattered pituitary parenchyma cells, a comment also made in earlier descriptions. We could not be certain that the granuloma cells were true histiocytes and we agree that the picture may represent a primary unexplained breakdown of the pars anterior. But this hypothesis is not easy to reconcile with the presence of the additional discrete granulomas in the pars nervosa and capsule of case 1 and in the stalk in case 2. However, it is conceivable that these might represent a reaction to products of destruction diffusing out from the pars anterior.

The peculiar inclusion bodies must be interpreted with care. The anisotropic and hæmatoxyphilic materials might be products of disintegrated pituitary cells. On the other hand, the latter certainly resemble the basophilic giant-cell inclusions described by Schaumann (1941) in the tonsils and lymph-nodes in sarcoidosis, and Tillgren (1935) illustrates similar bodies in the pars nervosa from a case of diabetes insipidus due to sarcoidosis. The sarcoid hypothesis is not borne out by the results of reticulin impregnation in our cases. The major part of the lesion fails to show the closely-knit network of reticulin fibres arranged around clear-cut follicles typical of sarcoidosis. In addition, basophilic giant-cell inclusion bodies have been found by Schaumann in many different types of granuloma. Recently fresh light has been thrown on the non-specific nature of these basophilic inclusions by Grier *et al.* (1948), who observed them in the lesions of beryllium granulomatosis.

The adrenal lesion in case 1 is most striking and has only once before been described in this association (Oelbaum and Wainwright). It might be of similar hypothetical " infective " origin to the pituitary lesion, but it is hard to see why these two organs and no others should have been the seat of election for this major lesion. It might represent a hypothetical coincidental primary " breakdown " of these two endocrine organs. It is more reasonable to suppose that the adrenal atrophy resulted secondarily from an absence of supply of adrenocorticotropic hormone by the necrosed pituitary. The picture differs chiefly in its wealth of giant-cells from the classical adrenal atrophy of Simmonds's disease and Addison's disease. Vines (1949) illustrates similar giant cells in a case of primary adrenal atrophy and one of Oelbaum and Wainwright's cases also shows these adrenal changes. The tiny splenic collection of epithelioid cells, rich in enfolding reticulin fibres and devoid of the inclusion bodies seen in case 1, shows little affinity to the above lesions.

The granulomatous coronary arteritis in case 2 does not appear to be connected, histologically or ætiologically, with the pituitary lesion, but a search for a possible arteritis in the pituitary region showed a small granulomatous focus surrounding an arteriole in the stalk. The pituitary lesion, however, is unlikely to be ischæmic in origin, for apart from the patency of the vessels in all sections examined, the picture is totally different from that seen in any stage of the now well-known ischæmic necrosis described by Sheehan. An ætiological connection cannot be disproved. The finding of the minute epithelioid-cell collection in the liver of case 2, especially when added to the splenic collection in case 1, is disturbing. Presumably these findings in random sections represent a wide distribution in the liver and spleen respectively. Unexpected miliary "tubercles" in these organs are an occasional post-mortem finding but their appearance differs so greatly from the pituitary and adrenal lesions that we are unwilling to be forced by their presence into a positive diagnosis of atypical syphilis, tuberculosis or sarcoidosis of the pituitary gland.

*Our findings and discussion have led to no positive conclusion as* to the ætiology of pituitary giant-cell granuloma. Like previous observers we are tempted to interpret the histology in favour of a primary parenchymatous necrosis of the pars anterior of the pituitary rather than a destructive infiltration by an infective granuloma, but we are held back from full confidence in this interpretation by the finding of granulomatous lesions, admittedly differing in detailed morphology, in other parts of the body.

## SUMMARY

1. Two cases of giant-cell granuloma of the anterior pituitary are described in detail.

2. Widespread granulomatous lesions were found in other organs in both cases.

3. No conclusion was reached as to ætiology. Although tuberculosis, syphilis and sarcoid were all considered, none could be either accepted or rejected.

We wish to thank Mr E. V. Wilmot for the photomicrographs of case 1, and Mr R. S. Morgan for the photomicrographs and Mr J. F. C. Willder for the sections in case 2.

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615 . 9 : 547 . 564 . 3 (*p*-nitrophenyl diethyl thiophosphate)

# POISONING BY *P*-NITROPHENYL DIETHYL THIOPHOSPHATE (E.605): A CONTRIBUTION TO THE STUDY OF ANTICHOLINESTERASE COMPOUNDS

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(PLATES X AND XI)

THIS paper describes the pathological changes in rats poisoned by E 605 (*p*-nitrophenyl diethyl thiophosphate). The synonym, Parathion, widely used in the United States, has the disadvantages of being a trade name and of lacking complete specificity. The identification number, E 605, was used by Schrader (1947), who first prepared this compound, and is the symbol by which it is known in this country.

E 605 is characterised by its power to inhibit cholinesterase (DuBois *et al.*, 1949), and a number of related compounds share this property. One of these, diisopropyl fluorophosphonate (D.F.P.), has been extensively used in physiological research and two others, tetraethyl pyrophosphate (T.E.P.P.) and hexaethyl tetraphosphate (H.E.T.P.), have been tried therapeutically in man for the treatment of post-operative paralytic ileus and myasthenia gravis. E 605 is a relatively stable and highly potent insecticide, particularly effective against aphids, and its increasing use in agriculture has shown the need for further information on its mammalian toxicity.

## METHODS

More than 150 adult male and female albino rats (wt. 150-200 g.) of mixed origin were used. E 605 was dissolved in ethyl alcohol, ethyl lactate, propylene glycol or arachis oil before injection. A micrometer syringe was used and the size of the inoculum never exceeded 0·2 c.c. The toxicity of E 605 varies with different samples, the LD 50 ranging from 1·0 to 15 mg. per kilo. This inconsistency can be attributed at least in part to the varying proportions of stereo-isomers of differing toxicity in the samples. Subcutaneous, intravenous and intraperitoneal routes were used for injection. Prolonged poisoning was induced by repeated injections of E 605, using the clinical state of the animal as a guide to dose and frequency of injection. The usual procedure was to give a dose of one-half the LD 50 dose and subsequently to inject one-tenth the LD 50 dose whenever symptoms disappeared. Prolonged poisoning was also induced by incorporating E 605 in the diet.

Tissues taken at post mortem were fixed in a variety of fixatives depending on the histological method to be employed. Heidenhain's Susa was the routine fixative, but Helly's and Carnoy's fluids and formol-saline were also used. Tissues were paraffin-embedded. Ehrlich's acid-hæmatoxylin and eosin was used as a routine stain and was the only method used for lung, striated muscle, heart muscle, thyroid, adrenal, pituitary, ovary and testis. For other tissues additional staining methods were employed. The salivary and lachrymal glands, pancreas and other organs of the digestive system were fixed in Susa or Helly's fluid and stained by toluidin blue, Lillie's modification of Giemsa, Heidenhain's azan, Mayer's mucicarmine, McManus's periodic acid leucofuchsin technique and a combination of Weigerts's iron hæmatoxylin, Altmann's acid-fuchsin and aurantia. Bowie's modification of Bensley's neutral gentian method was used to examine the islet tissue of the pancreas. Lymphoid tissue was stained by Lillie's modification of Giemsa. Heidenhain's azan was used for the kidney, and the liver was stained with toluidin blue and Best's carmine. In the examination of the Harderian gland, frozen sections were cut after formol-saline fixation and gelatin embedding. These were stained for fat with Sudan IV.

The brain and spinal cord were fixed in Carnoy's fluid. Special precautions are necessary to obtain adequate fixation of the spinal cord in rats. After several trials the following procedure was adopted. Immediately after death the spinal column was opened in the cervical and lumbar regions and Carnoy's fluid was injected through the cervical opening until the fixative ran out uncoloured from the lumbar opening. At least 30 c.c. of fixative were used. The whole bony column with the cord *in situ* was dissected free from the musculature and immersed for one hour in Carnoy's fluid. The hardened cord was then dissected free from the vertebral column and placed in fresh Carnoy for a further five hours. If these precautions are not observed, chromatolysis and œdema of the anterior horn cells are commonly found in both experimental and control animals.

## EXPERIMENTAL OBSERVATIONS

### *Symptoms*

Within a few minutes of the subcutaneous injection of a lethal dose of E.605 the demeanour of the animals is changed. They sit quietly, hunched up, ruffled and apprehensive. Salivation commences and is evidenced by repeated swallowing and by grooming of the whiskers. The condition progresses and the animals are prostrated. After ten minutes the rat lies full length on its belly with extended head and flexed limbs. Fine fibrillary twitchings give the rat's coat a peculiar shimmering effect. Occasional coarse tremors interrupt the twitchings and shake the animal violently from side to side for a few seconds before it subsides again on to the floor of the cage. Chattering of the teeth is clearly audible. Salivation is excessive, and unswallowed mucinous saliva surrounds the mouth and nose. Lachrymation, exophthalmos, mucous diarrhœa and the frequent passage of small amounts of urine are other symptoms that develop within ten minutes of the injection of E.605. Respiration is embarrassed and noisy and the animal may extend its neck and forelimbs in a jerky manner in forced respiration. Death is usually sudden and is preceded by severe coarse tremors. Death may occur at any time from a few minutes to several hours after the injection. If the animal survives, the symptoms slowly decrease in severity and after a few hours there is complete recovery. Although apparently normal it is now, however, highly susceptible to E.605, and a very small dose, which would be without effect on a fresh animal, will provoke severe symptoms and even death.

By giving a series of injections or by adding E.605 to the diet the rat may be rendered chronically ill, with symptoms which include tremors, diarrhœa and salivation for periods up to several weeks. If the symptoms are prolonged beyond a few hours the condition of chromodacryorrhœa—the shedding of pink tears—is a constant feature. These dry and encrust around the protruding eyes or fall on the coats of the cage mates, which then take on a brownish-red tinge. These more prolonged symptoms do not prevent the animal from eating and moving about its cage.

### *Post-mortem findings*

In acute poisoning twitching of both muscles and limbs continues up to 30 minutes after death. *Rigor mortis* is slow in onset. The hair around the mouth and nose is matted with mucus and often red crusts line the eyelids. The trachea contains a slight excess of mucus; the lungs are expanded and hyperæmic. The heart is in ventricular systole and the vessels are normal. The stomach is often contracted but usually contains some food. The small and large intestine contain only bile-stained fluid thickened with mucus. The intestine is often contracted, but intussusception and volvulus have not been seen. There is no hæmorrhage or ulceration in stomach or intestine. The pancreas is conspicuously pink and the same tinge may be seen in the parotid glands, but the other salivary glands and the orbital glands are macroscopically normal. The liver may be congested. The kidneys and adrenals are normal and the bladder is empty. The lymph nodes and thymus are normal but the spleen is contracted and bright red. The vessels of the meninges are congested, but no gross change can be seen in the brain or spinal cord.

### *Histological findings*

The occurrence of recognisable anatomical lesions depends on the severity and duration of symptoms. If a large dose of E.605 of the order of ten times the LD50 dose is given, death occurs within thirty minutes and no histological changes can be detected. The more highly active anticholinesterases—tetraethyl pyrophosphate (T.E.P.P.) and E.600—produce symptoms of only about half-an-hour's duration and again no lesions are produced unless the symptoms are prolonged by repeated injections. If symptoms persist for more than three or four hours, structural changes occur. These are found particularly in the salivary and lachrymal glands and in the acinar portion of the pancreas, but there is a difference in the ease with which lesions can be produced in different glands. Changes are most easily induced in the lachrymal and submaxillary glands and much less readily and less consistently in the parotid glands and pancreas. Changes in the spleen and thymus are found only when symptoms have persisted for some days. The histological changes in the salivary and lachrymal glands and in the pancreas produced by prolonged poisoning of several days' or weeks' duration are the same as those occurring in acute poisoning when symptoms have lasted for three or four hours.

The gross anatomy of the salivary and orbital glands of the rat has been described by Greene (1935), and the comparative histology of the salivary glands by Krause (1921) and Stormont (1932). Fekete (1941) has given a very complete account of the normal histology of the salivary and orbital glands in the mouse, but there is no corresponding account of the normal structure of these glands in the rat. The normal histology of the different glands is therefore discussed in sufficient detail to enable a comparison to be made with the glands of poisoned animals.

*Submaxillary glands.* These are two prominent structures in the ventral cervical region in contact along the mid-ventral line from the level of the hyoid bone almost to the manubrium sterni. They are compound tubulo-alveolar glands divided into a number of lobules by connective tissue septa. They have been described by Ranvier (1886) and Loewenthal (1893-94) as serous, and Honda (1926-27) has reported the presence of some sero-zymogen cells. Stormont (1932) has described the alveolar cells as special serous cells differing from the sero-zymogenic type and both cytologically and functionally ill defined. As yet there is nothing to add to Stormont's summing up.

The duct system is extensive. The main duct, lined by pseudostratified columnar epithelium, and the interlobular ducts, lined by columnar epithelium, are unaffected in prolonged E.605 poisoning. The intralobular ducts are striated tubules lined by rodded epithelial cells. They have centrally located nuclei with basal cytoplasmic striations radially arranged. The intralobular ducts divide into terminal tubules lined by tall columnar epithelial cells which protrude into and appear to fill the lumen. These cells have basal nuclei, often flattened, and a large amount of cytoplasm which stains with eosin to a variable degree. Some of the cells contain granules and probably correspond to the sero-zymogen cells described by Honda. In E.605 poisoning the number of granules increases greatly, all the cells of the terminal tubules and many of those of the intralobular ducts becoming distended with them. These granules are best stained by Altmann's acid-fuchsin.

At the same time changes are found in the alveoli. The special serous cells which form the alveoli are pyramidal in shape, with a large oval darkly-staining nucleus near the base of each. The cytoplasm is basophilic, with concentration of basophilic material towards the base of the cell. The cytoplasm does not contain any zymogen granules. In E.605 poisoning there is early and slight reduction in

POISONING BY E 605

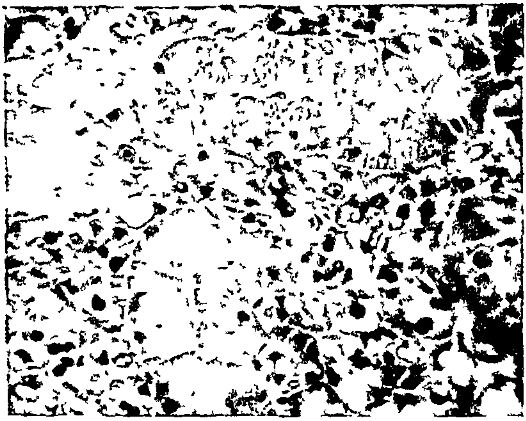

FIG. 1.—Submaxillary gland of male rat, killed after 3 subcutaneous injections daily for 2 days of E 605 in arachis oil. Collapse of alveolar structure and vacuolation of alveolar epithelium. Increase in granules in tubules. Altmann's acid fuchsin. ×470

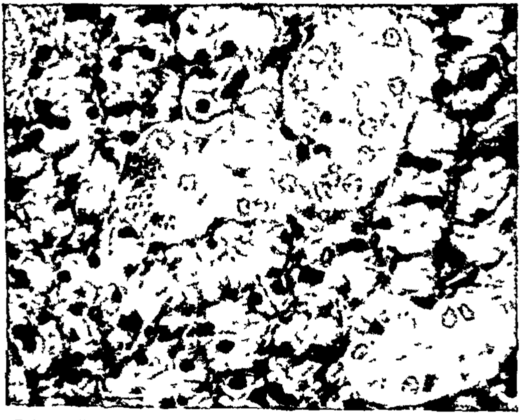

FIG. 2.—Submaxillary gland of a normal male rat for comparison with fig. 1. The dimensions of the normal alveoli and tubules are shown. The presence of granules in some of the tubular cells is illustrated. Altmann's acid fuchsin. ×470

cytoplasmic basophilia. Vacuoles appear in the cytoplasm, first in isolated cells, then in groups of cells: finally every cell is involved. The alveolar structure collapses and the shrunken alveolar cells become packed together. The alveolar tissue becomes reduced relatively to the tubular part of the gland, which is now prominent and strongly fuchsinophilic (fig. 1; *cf.* fig. 2). In such glands postmortem autolysis is rapid and accentuates these changes.

*Major sublingual glands.* These are paired structures closely applied to the antero-lateral surface of the submaxillary glands and made up of a single lobe divided into lobules by connective tissue septa. They are mucous glands and are stimulated to very active secretion by E.605. The only histological change is a slight decrease in depth of staining of the mucin within the cells. Mucus can be seen within the ducts but the duct epithelium is unaltered.

*Parotid glands.* These glands spread diffusely on either side over the ventro-lateral surface of the neck and reach the shoulder. Each consists of a number of elongated tubules. They are sero-zymogenic glands similar to the acinar portion of the pancreas. The serous cells of the secretory alveoli are pyramidal and their basal cytoplasm is strongly basophilic, with vertical striations. The nucleus is round or oval and relatively large, and on the apical side of the nucleus coarse zymogen granules fill the cell. In E.605 poisoning there is loss of both basophilia and zymogen granules. The cells shrink and the acini become deformed, and small vacuoles appear in the cytoplasm. Many nuclei become pyknotic and hyperchromatic. As a result of these changes there is loss of definition of the alveolar cells and the regular glandular pattern is also lost (fig. 3). The duct epithelium is entirely unaffected. These changes are produced only after prolonged and severe poisoning and are always less marked than those in the submaxillary gland.

*Lachrymal glands.* Each lachrymal gland consists of two separate parts. The intra-orbital gland is a small triangular structure covering the posterior angle of the orbit beneath a connective tissue sheath. The extra-orbital gland is a well-defined discoid structure lying below and slightly in front of the ear and close to the anterior border of the parotid gland. Both parts, although anatomically separate, are histologically identical. They are tubulo-alveolar in structure and composed of small lobes divided into lobules by fibrous tissue. The ducts are lined by low cuboidal epithelium lacking basal striations. The alveoli are large and loosely arranged and the component cells are also large and bulging, and variable in shape. They are columnar, pyramidal or hour-glass shaped and their nuclei are large and prominent, with a well-defined nuclear membrane and one or more nucleoli. Large nuclei and double nuclei are common. The cytoplasm is strongly basophilic and contains somewhat ill-defined basophilic granules. In E.605 poisoning there is no obvious change in the degree of basophilia, but vacuoles appear in the cytoplasm (fig. 4).

They are most frequent in the apical portion of the cells, where they are often multiple. They are also found in the basal region and may even involve the nucleus. The cells tend to shrink, with the appearance of better-defined lumina, and the large nuclei disappear. As a result there is greater uniformity of cellular structure except where the vacuolation has progressed to produce hydropic degeneration of cells or groups of cells. Minor degrees of vacuolation are not uncommon in normal rats that have been killed by chloroform inhalation but it is rare in rats killed by stunning.

*Harderian gland.* This is a large horse-shoe-shaped gland lying behind the eyeball and almost completely encircling the optic nerve. It produces the red tears that are a feature of E.605 poisoning. It is a tubulo-alveolar gland, and both tubular and alveolar cells are extremely pale-staining, with absence of basophilia and very little affinity for acid dyes. But the appearance of frozen sections stained with hæmatoxylin and neutral red is striking. The cytoplasm is completely filled with secretory granules of a lipoidal nature. Small pale round nuclei lie at the base of the cells. In poisoning with E.605 there is evidence of increased secretion. The alveoli and ducts become filled with fat globules outlined by brownish granular material, which is the precipitated porphyrin responsible for the red colour of the tears. There may be some slight reduction in the depth of the cells, with corresponding widening of the alveolar and tubular lumen, but there is no reduction in lipoid granules in the residual cytoplasm. There is no change in the duct epithelium.

*Pancreas.* The first change in the pancreas in E.605 poisoning is a reduction in the number of zymogen granules in the acinar cells. Before this reduction has become extreme, vacuoles appear. They are round and clear, and range from 1 to 10 $\mu$ in diameter. They may occupy any part of the cytoplasm, but are more frequently apical than basal. A single cell may contain several small vacuoles. In some cases vacuoles are present in all the acinar cells; in others they occur in foci involving several groups of cells (fig. 5). When focal in distribution they are frequently confined to the area immediately surrounding an islet of Langerhans. There is a tendency in this region for the zymogen granules to persist, so that vacuoles are found in acinar cells which also contain many zymogen granules. The pancreatic ducts are unaffected and the islet tissue is normal in all respects.

*Intestinal canal.* The squamous epithelium which lines the cardiac end of the rat's stomach is unaffected in E.605 poisoning. Of the columnar epithelium, with many glandular elements, which lines the pyloric end, only the mucus-secreting goblet cells show signs of increased activity. The gastric mucosa is coated with mucus, which still stains strongly in the goblet cells. The duodenum, ileum and large intestine are empty and the mucosa is coated with mucus. The goblet cells stain strongly for mucin and seem to be unaffected by

POISONING BY E 605

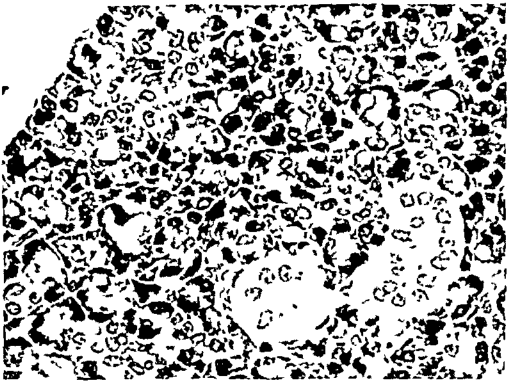

FIG. 3.—Parotid gland of male rat killed after 2 subcutaneous injections daily for 2 days of E 605 in ethyl lactate. Distortion of alveolar structure and vacuolation of alveolar epithelium. Hæmatoxylin and eosin. ×470.

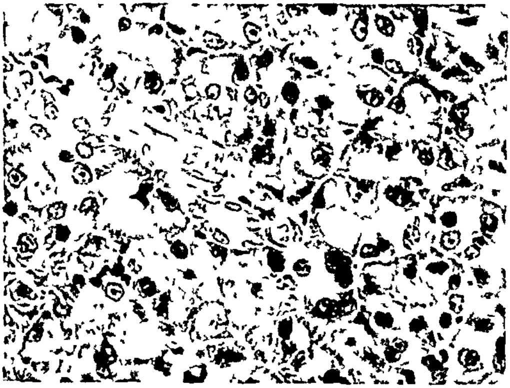

FIG. 4.—Lachrymal gland of male rat, killed after 2 subcutaneous injections daily for 2 days of E 605 in ethyl lactate. Vacuolation of alveolar cells. Hæmatoxylin and eosin. ×470.

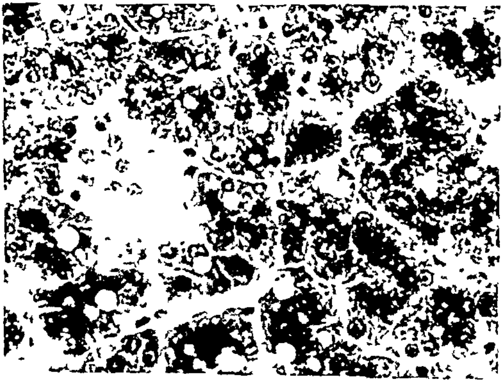

FIG. 5.—Pancreas of male rat killed after daily oral administration of E 605 in arachis oil for 14 days. Zymogen granules still present. Vacuolation of acinar cells. Altmann's acid fuchsin. ×470.

their increased secretory activity. No ulcers, hæmorrhages or necrotic lesions are seen in either stomach or intestine.

*Lymphoid tissue.* The spleen, thymus, and abdominal, thoracic and cervical lymph nodes were examined. No changes are found in acute poisoning with E.605 where symptoms have lasted less than four hours. In prolonged poisoning of 3 or 4 days' duration the spleen shows a relative reduction in white pulp. Lymphocytes are diminished in number and nuclear debris appears in the germinal centres, which later become empty and acellular. After prolonged poisoning lymphocytes disappear from the red pulp also, leaving behind the reticular and fibrous tissue. In the thymus the changes produced by prolonged poisoning affect particularly the cortical lymphocytes. Here the reduction in the number of lymphocytes may be so great that the distinction between cortex and medulla is lost, except in extreme cases where the lymphocytes are more densely packed in the medulla than in the cortex, a reversal of the normal picture. No consistent changes are found in any of the groups of lymph nodes.

*Other tissues.* These can be considered as a group based on the slight or negative findings rather than on any particular anatomical basis. The *lungs* do not show any marked change. Capillary congestion is usual, with minor degrees of œdema and some associated collapse. In some cases the bronchial and bronchiolar mucosa is much folded, suggesting ante-mortem spasm. This finding, however, is inconstant and inconclusive. The liver does not show any pathological change. After a single injection of an LD50 dose of E.605 the liver glycogen disappears completely and does not reappear in the liver of survivors until they have completely recovered symptomatically. The *thyroid*, *pituitary* and *adrenal glands*, examined only after routine staining with hæmatoxylin and eosin, show no lesions or abnormalities of any sort. The *kidneys, bladder, ovaries, testes, brain, spinal cord, heart muscle, smooth muscle* and *striated* (*skeletal*) *muscle* present normal histological appearances. The myoneural junctions, examined by the gold, silver and methylene blue methods, show no consistent changes in the poisoned animals.

## DISCUSSION

In E.605 poisoning, whether acute or prolonged, and indeed in poisoning by other agents with anticholinesterase activity, most of the important organs of the body are free from well-defined pathological lesions which can be held accountable for the death of the animal (see also Forssling, 1948, Jones and Landing, 1948, DuBois *et al.*, 1949). On the other hand, changes in the exocrine glands are a constant feature of prolonged E.605 poisoning. Similar changes have been described before in other conditions. Beattie and McDonald (1933) found that a single injection of acetylcholine produced small cytoplasmic vacuoles in the cells of the lachrymal glands of cats. Vacuolation of the acinar cells of the pancreas has been produced by the injection of pilocarpine (Mouret, 1895), by electrical stimulation of the vagus (Babkin *et al.*, 1909) and by repeated injections of acetylcholine (Leblond and Sergeyeva, 1944; Tucker, 1948). The persistence of granules in the acinar cells lying immediately around the islets to form halos of unexhausted cells has been described occasionally in the normal pancreas (Jarotzky, 1899; Dale, 1905), and Sergeyeva (1938) has shown that this condition can be induced by vagal stimulation.

Hillarp (1949) has produced changes in the submaxillary glands of rats by sympathetic stimulation and by provoking intense secretion by feeding on dry wheat starch. He describes shrinking of the alveolar-cell acini and some vacuolation, but found a reduction in granules in the tubular part of the submaxillary gland.

Ehrich and Seifter (1948) have described changes in the salivary glands and pancreas of rats following the injection of colchicine and a number of other compounds. They attributed these changes to the so-called alarm syndrome, which is part of the adaptation syndrome of Selye (1946), a syndrome embracing also chromodacryorrhœa and lymphoid hypoplasia. In view of the similar changes produced by prolonged E.605 poisoning it is possible that the conditions found by Ehrich and Seifter may be due to the excessive production or accumulation of acetylcholine. If this could be established it might then be possible to define more specifically some aspects of the alarm reaction.

The extent of the changes described in the lymphoid tissue is difficult to assess because these tissues are extremely variable in apparently normal animals. But the changes seen in the thymus and spleen in E.605 poisoning are both consistent enough and severe enough to be attributable to the action of E.605. Similar changes have been described by Jones and Landing (1948) with two phosphine oxide anticholinesterases, but they found in addition marked lymphocytic degeneration and hypocellularity in the regional lymph nodes and Peyer's patches of the intestine. They compared these findings with those produced by nitrogen-mustard vesicants which, as Thompson (1946-47) has shown, can also inhibit cholinesterase activity.

Chromodacryorrhœa and the histological evidence of hyperactivity of the Harderian glands are interesting features of E.605 poisoning. The shedding of red tears by the rat was first described by Freud (1933) following the injection of muscarinic drugs, and Tashiro *et al.* (1940) showed that red tears also result from the intraperitoneal injection of acetylcholine. Thompson demonstrated that chromodacryorrhœa develops two or three days after the application of vesicants such as mustard gas and nitrogen mustard, and attributed this action to the inhibition of cholinesterase activity at cholinergic nerve endings. Burgen (1949) has developed a test in which the appearance of red tears in rats is used as a means of assessing the potency and duration of action of anticholinesterase drugs. The red pigment in the tears is probably the porphyrin found in the Harderian glands of rats by Derrien and Turchini (1924). The chief component is a protoporphyrin (Fischer and Orth, 1937; Towbin *et al.*, 1945) but McElroy *et al.* (1941) describe a coproporphyrin as a second component.

A consideration of the pharmacological action of acetylcholine enables one to predict most of the findings in this paper, although other changes also might reasonably have been expected. Intussusception and volvulus have been produced in young guinea-pigs by the injection of pilocarpine (Flory and Allen, 1949) but have not been observed in rats subjected to prolonged poisoning by E.605. Gastric and intestinal ulceration has been looked for but not found. Koppanyi (1948) has suggested that acetylcholine poisoning might produce hæmorrhages in the grey and white matter of the central nervous system, accompanied by neurone depletion, diffuse gliosis, and the formation of glial nodules and scars. This is in line with the present tendency to extend the scope of the action of cholinesterase and to consider its relation to tissue oxidation. In E.605 poisoning, no lesions of any kind were found in the brain or spinal cord.

The histological changes produced by E.605 seem to result from the action of acetylcholine liberated at cholinergic nerve endings and *persisting in the absence of the specific enzyme which normally inactivates it*. But the development of pathological lesions and their severity are dependent on the duration of symptoms of E.605 poisoning. When large doses are given, death occurs within thirty minutes and histological changes are not found. With more highly active anticholinesterase agents such as tetraethyl pyrophosphate (T.E.P.P.) and E.600, symptoms appear quickly and, if death is not immediate, rapidly disappear without leaving any histological changes. Lesions in the pancreas and salivary glands are found only when the action of E.605 is prolonged for at least three hours. Other workers have met with similar difficulty in producing lesions by the injection of acetylcholine or by vagal stimulation. Injections of acetylcholine must be repeated at ten-minute intervals for several hours and the vagus must be stimulated continuously for some hours before vacuolation of the pancreas occurs.

## SUMMARY

1. Rats poisoned with E.605 and surviving for at least 3 hours show the symptoms of lachrymation, excessive salivation, chromodacryorrhœa, muscular weakness, diarrhœa and embarrassed respiration.

2. The most constant pathological finding in animals dying after three hours is in the submaxillary glands, where the acinar cells of

the alveolar portion are vacuolated and shrunken and th ecells of the tubular portion filled with eosinophilic granules.

3. Vacuolation also occurs in the parotid and lachrymal glands and in the acinar portion of the pancreas. The sublingual and Harderian glands show evidence of increased secretory activity but no degenerative changes.

4. The thymus and spleen are depleted of lymphocytes but the lymph nodes are normal.

5. The changes are attributed to the inactivation in the body of cholinesterase by E.605, with resulting accumulation of acetylcholine at cholinergic nerve endings.

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of 1 per cent. trypan blue being given intravenously. The rabbit appears to be about one-half as sensitive as the rat, so that a substance having an end-point of 1 : 20,000 in the latter animal will show its final response at 1 : 10,000 in the former. The permeability response, as evidenced by a patch of blue colouration in the skin, begins in 1-3 minutes and reaches its maximum in 20-30 minutes, when it is read. In control experiments 0·9 per cent. NaCl freshly prepared by diluting stock 9 per cent. saline 1 in 10 was injected intradermally.

Leucocytic emigration was likewise demonstrated by the method of Menkin. Rats were again used but no dye was administered. The abdomen was shaved under anæsthesia and the test solution injected intradermally. Thirty minutes later the animal was killed and the piece of skin cut out, fixed in Zenker's fluid, cut in paraffin and stained with Ehrlich's hæmatoxylin and eosin. A positive response was taken as unmistakable polymorph emigration shown by the leucocytes passing between the endothelial cells and by their presence clustered around the vessels. Although emigration was confined to polymorphs, some lymphocytes and monocytes were often found in the vicinity of the involved vessels.

The third biological effect of the preparations studied, swelling of the capillary endothelium, was demonstrated in the same sections. This phenomenon, although not stressed by previous workers, was usually well marked in the material of this investigation. Estimation of both these properties is only roughly quantitative.

The peptic digest of fibrin was prepared by the method of Cullumbine and Rydon, the digestion mixture being brought to *p*H 2 before incubation for 3 hours at 37° C. and the fibrin being triturated with small quantities of water before digestion to facilitate its suspension. Deproteinisation with acetone and pyridine was carried out according to the directions of the same authors. B.D.H. blood fibrin and Benger's pepsin 1 in 10,000 were used.

Charcoal adsorption and elution were also performed by the method of Cullumbine and Rydon. Fuller's earth adsorption and elution were carried out by the method of Duthie and Chain except that acetone/pyridine deproteinisation was substituted for the first pressure filtration and centrifugation for the second. Trypsin digestion was carried out with B.D.H. crude trypsin powder.

Paper partition chromatography was performed by the techniques of Gordon, Martin and Synge as modified by Dent (1947). Electrodialysis was undertaken in a four- or five-celled apparatus made from a perspex cylinder by Mr A. W. Hemmings of the department of chemical pathology, the cells being separated by membranes of cellophane or formalin-hardened parchment. Ionophoresis was carried out in a trough of the type described by Consden *et al.* (1946), with lead electrodes and glass-wool packing instead of silica gel, the apparatus being kindly supplied by Miss Nicholas.

Hydrolysis of peptides to the amino-acid stage was performed by adding an equal volume, generally 1 ml., of concentrated HCl to the test solution and heating at 100° for 36 hours in a boiling tube fitted with a cold finger. Free ammonia nitrogen was determined with Nessler's reagent and total nitrogen by the micro-Kjeldahl titrimetric technique as described by Hawk *et al.* (1947). Chain lengths of the peptides were determined by free amino-nitrogen determinations before and after acid hydrolysis (Winnick, 1944). The nitrous-acid and ninhydrin-$CO_2$ methods of Van Slyke were used. In the former a correction was made for ammonia present in the preparations by determining free ammonia nitrogen by the direct Nessler method and then estimating the pressure of nitrogen gas yielded by this quantity of ammonia by reference to the pressure yielded by a known solution of ammonium sulphate on submitting it to the nitrous acid procedure. In every case the free carboxyl nitrogen of the acid hydrolysates was determined by the ninhydrin-$CO_2$ method (Van Slyke *et al.*, 1941) and found to agree with amino nitrogen determined by the nitrous-acid method (Van Slyke, 1929) after the ammonia nitrogen correction had been made.

## RESULTS

### *The permeability response*

Menkin (1937) found that whereas his extracts gave a homogeneous patch of dye-staining in the permeability test, histamine gave a ring-like response. In the rat, dropping the concentration of histamine to below the 0·05 per cent. mark replaces the ring reaction given by higher concentrations with a patch response. Moreover, when the partially purified peptide material is increased in concentration to above 1 per cent. the ring type of dye staining makes its appearance. Indeed, it is possible to obtain by electrodialysis a fraction of the peptide mixture utilised in this investigation which gives a ring type of response all the way down to concentrations of 0·05 per cent.

The effect on permeability of glutathione, adenylic acid, creatine, crystalline penicillin "G" and pepsin was also tested. At concentrations of 1 per cent. vasoconstriction and blanching occurred, sometimes followed by a delayed and faint ring-like zone of dye staining. At concentrations of 0·1 per cent., no response of any kind could be detected. Irritants such as acetone, pyridine, turpentine, sulphonated alcohol ("Teepol") and 0·2 *N* sodium hydroxide gave a similar constrictor response; at dilutions ranging from 1 to 0·1 per cent., no reaction was visible. This contrasts with partially purified peptide preparations which under the same conditions yielded a well-marked increase in capillary permeability at concentrations of 0·005 per cent.

### *Attempts at purification of peptic digest of fibrin by adsorption*

The crude digest was adsorbed and eluted off charcoal as described by Cullumbine and Rydon, but no purification could be achieved, the activity of the starting material usually being distributed equally between the non-adsorbed residue and two or three of the fractional eluates. Charcoals employed consecutively were B.D.H. activated charcoal, Light's activated charcoal with and without preheating in a crucible, and "Norit". The crude digest was then adsorbed and eluted with B.D.H. adsorption fuller's earth. The observation by Duthie and Chain that the active material was almost quantitatively adsorbed was confirmed, but elution yielded only a small quantity of partially insoluble material only slightly more active than the original digest. It was found that the ammonia and pyridine eluates were of approximately equal activity, in contradistinction to the results of Duthie and Chain, who found the bulk of the activity in the latter fraction.

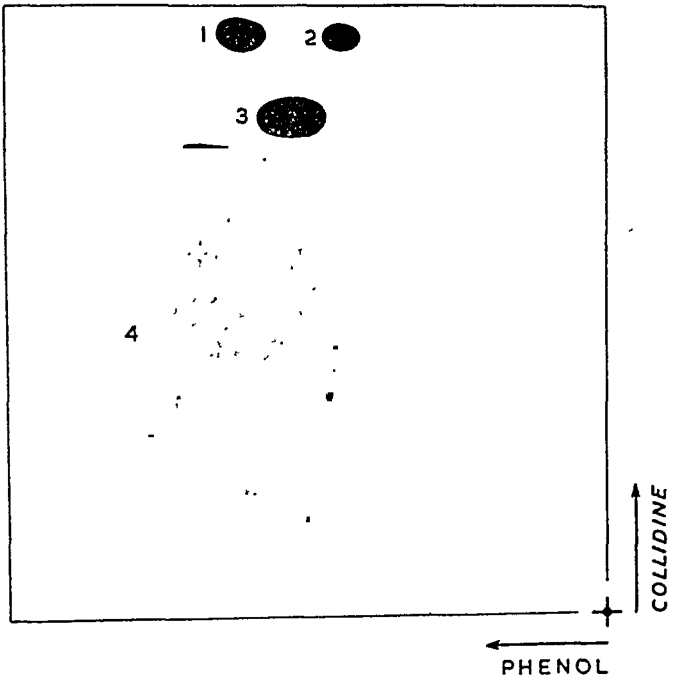

FIG. 1.—Chromatogram of peptic digest of fibrin. 1. Phenylalanine. 2. Tyrosine 3. Leucine or isoleucine or both. 4. Peptide streak.

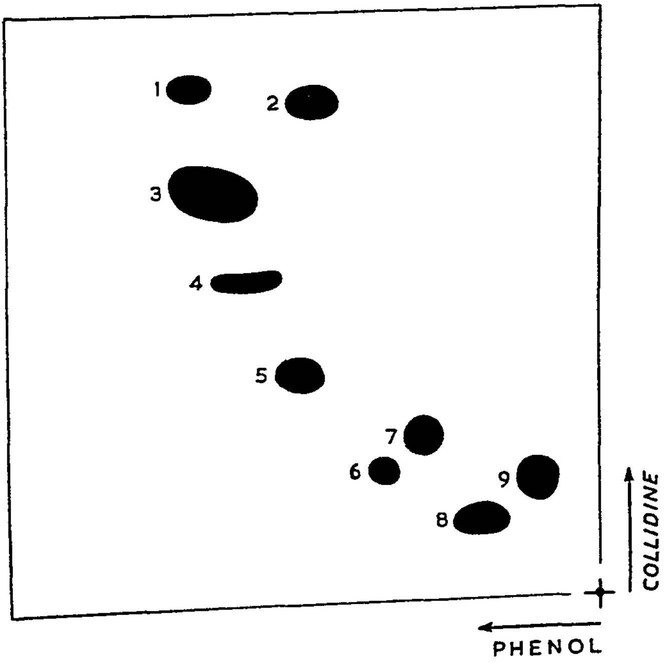

FIG. 2.—Chromatogram of amino-acids liberated by acid hydrolysis of the active principle of goat turpentine pleural exudate. 1. Phenylalanine. 2. Tyrosine. 3. Leucine or isoleucine or both. 4. Valine. 5. Histidine. 6. Glycine. 7. Serine. 8. Glutamic acid. 9. Aspartic acid.

Since all the other common adsorbents had been used without success by Duthie and Chain, Cγ alumina gel (Wilstatter), which had not been previously used, was tried, but no concentration of activity was achieved.

### *Attempted purification by ion exchange*

A centrifuged solution of crude digest was run through the cation exchanger Amberlite I.R.C. 50 and the effluent plus washings then run through the anion exchanger Amberlite I.R.4.B. No separation of the active principle was achieved; the HCl eluate from the cation and ammonia eluate from the anion exchangers consisted largely of salts. Since the effluent actually lost some activity, a degree of adsorption was indicated.

The amphoteric alumina column was also used (Savory and Moore's standardised alumina). Acid-washed, ammonia-washed and untreated columns yielded no real concentration of activity. There was some adsorption of highly coloured material at the top of the column which could be eluted off both by acid and alkali and which contained about 1/10th of the activity of the remaining digest. Fractional effluents all had the same order of activity.

### *Attempted purification by paper partition chromatography*

Using the same solution, one-dimensional paper-strip chromatograms were run in phenol, collidine-lutidine, butanol and butanol-acetic acid and developed with ninhydrin. No visual separation was achieved, but when the strips were cut up into segments and eluted (Dent), the activity was found to reside in the ninhydrin-reacting section, except in the collidine-lutidine strips, where no colour developed after treatment with ninhydrin. That the activity was not due to ninhydrin was shown by repeating the experiment with strips untreated with this compound.

Two-dimensional chromatograms of the same solution were run on paper squares, first in phenol then in collidine-lutidine. On developing with ninhdrin the digest was seen to contain free tyrosine, phenylalanine, and either leucine or isoleucine or both. In addition the chromatogram showed a broad streak giving a fainter blue with ninhydrin (fig. 1). This streak occupied the position taken up by higher peptides in these solvents. An undeveloped was superimposed on a developed square and pieces cut out corresponding to the ninhydrin-reacting areas and to the control areas. These paper segments were then eluted as before and the eluates tested for biological activity. It was found that all such activity resided in the streak occupying the position taken up by higher peptides and that the amino acid and control spots were inert.

For visual separation attempts 2·5-5 mg. of material were used, for elution experiments 5-10 mg. in a volume of 25-50 μl.

### *Water- and alcohol-soluble fractions of crude digest*

All the experiments so far described were performed on the completely water-soluble material obtained by centrifuging or filtering an aqueous solution of the crude digest. The water-insoluble material comprises about 10 per cent. of the whole digest by dry weight and corresponds to the alcohol-soluble fraction mentioned by Dekanski (1949). It is practically insoluble in water, but in strongly alkaline solution gives a fine suspension exhibiting birefringence of flow. Prolonged shaking with ethyl, methyl and butyl alcohol produces a yellow solution. The most rapid extraction was achieved with a mixture of absolute ethyl alcohol and ether in equal volumes. On shaking this extract with dilute acid, the yellow colour stayed in the alcohol/ether phase ; on similarly shaking with aqueous alkali, the colour moved into the aqueous phase. The colour of the water-soluble portion of the whole digest does not move into alcohol or ether from either acid or alkaline aqueous solution. In the capillary-permeability leucocyte-emigration and endothelial-swelling tests, both water- and alcohol-soluble fractions have the same order of activity; the latter, if anything, being a shade more potent.

Two-dimensional paper chromatography yielded information about the nature of the alcohol-soluble fraction. When an aqueous suspension of the material is run in phenol and collidine-lutidine the resulting pattern on development with ninhydrin is practically identical with that given by the water-soluble fraction. Thus the alcohol-soluble fraction contains free tyrosine, phenylalanine and leucine and higher peptides. This chromatogram pattern was obtained after repeated water washes, the sediment then being resuspended and run. It was concluded from these experiments that the alcohol-soluble fraction is composed of part of all the constituents of the whole digest bound up in some form of matrix, possibly of a lipoid nature. In view of this and because of the solubility characteristics of this fraction it was decided to confine further studies on the peptic digest of fibrin to the water-soluble portion.

### *Ammonium sulphate fractionation*

When a 2-5 per cent. aqueous solution of water-soluble digest was brought to 75 per cent. saturation with ammonium sulphate a dense precipitate appeared, part of which was resinous in appearance and floated to the surface. No further precipitate appeared on increasing the concentration of salt to 100 per cent. saturation. This precipitate has 5-10 times the activity of the starting material in the permeability test and the supernatant has none. To confirm that this latter result was not due to a masking effect exerted by the high concentration of ammonium sulphate this supernatant was de-salted by prolonged electrodialysis, after which it was found to be still inactive in the permeability test. In a typical pilot scale experiment 1 g. of water-soluble digest was dissolved in 25 ml. of distilled water and at room temperature 13·8 g. of ammonium sulphate added and the mixture shaken until dissolved. The precipitate was centrifuged down, drained and redissolved in water, 1 g. of starting material yielding 100 mg. of precipitate. Estimation of activity was carried out by the capillary permeability test, the precipitate being titrated side-by-side with some of the starting material which had been held back. The end-point of the precipitate was 0·005 per cent., that of the crude digest 0·05 per cent. Material was recovered from solution by evaporating to dryness on a water-bath under reduced pressure, or, in the case of small quantities, by drying *in vacuo* over $P_2O_5$.

The salted-out precipitate was more powerful in its effects on leucocyte emigration and endothelial swelling than the crude digest, giving positive results down to dilutions of 0·1 per cent., with massing of polymorphs around blood vessels at concentrations of 1-2 per cent. Two-dimensional paper partition chromatography revealed that the precipitate consisted of a peptide streak alone and that the supernatant contained the three free amino-acid spots of the original digest plus a smaller quantity of peptides.

### *Fuller's earth absorption of ammonium sulphate precipitate*

The ammonium sulphate precipitate was a dark brown amorphous substance readily soluble in water at *p*H 8. In its appearance and order of activity in the permeability test it resembled the material eluted off fuller's earth by Duthie and Chain, which contained all the activity of their digest. To investigate this point 50 mg. of the precipitate were shaken for 10 minutes with 100 mg. of fuller's earth and the adsorption repeated twice more. At the end of this time the supernatant contained no peptide material at all and was inactive in the permeability test. The supernatant, after adsorbing the whole crude digest with fuller's earth, was brought to 75 per cent. saturation with ammonium sulphate and the small quantity of salted-out material obtained was found to have an end-point in the permeability test of 0·1 per cent. Whether or not any of the inert peptides in the ammonium sulphate supernatant were adsorbed by fuller's earth could not be determined, as no sure method existed for their identification. Nevertheless, since all the active salted-out peptides were quantitatively adsorbed by fuller's earth, leaving, to all intents and purposes, nothing behind, it was concluded that the products of ammonium sulphate precipitation and of Duthie and Chain's procedure were largely the same.

### *Attempts at fractionation of the ammonium sulphate precipitate*

*Salting-out at different* pH *and ionic strength values.* For convenience in an attempt at fractional precipitation with ammonium sulphate the initial salting-out of the active material from the crude digest was done at 50 per cent. saturation instead of 75 per cent., the two precipitates having the same order of activity. One g. of precipitate was dissolved in 30 ml. of water and brought to 20 per cent. saturation with solid ammonium sulphate. This was centrifuged, the supernatant brought to 30 per cent. saturation and so on until a 60 per cent. saturation precipitate had been obtained, when the supernatant was discarded. This procedure was repeated at all *p*H levels from 2 to 9, the hydrogen-ion concentration being adjusted initially and between each precipitation with the aid of a glass electrode. At no saturation level at any *p*H was any further concentration of activity achieved, although the appearance of the precipitate varied from dark brown and resinous at 20 per cent. to pale yellow at 60 per cent. saturation.

The material precipitated between 40 and 50 per cent. saturation at *p*H 3·5 was then taken and re-precipitated twice at 50 per cent. saturation at the same *p*H. No concentration of activity resulted and the material could not be crystallised out from ammonium sulphate on prolonged standing.

*Organic solvent fractionation.* Fractional alcohol precipitation at 2° between 33·3 and 90 per cent. final concentration yielded no purification. A similar procedure with acetone at 2° between 66·6 and 90 per cent. also achieved no concentration of activity. Acetone or alcohol fractionation followed by ammonium-sulphate fractionation was likewise without effect.

*Electrodialysis.* When a solution of ammonium-sulphate precipitate at *p*H 3 was placed in the centre well of a four-celled electrodialysis apparatus with two anode compartments, the active principle of the capillary-permeability test appeared to migrate quantitatively to the cathode, leaving apparently inactive material behind. Yet the peptides recovered from the cathode cell had the same order of activity as the starting material, although comprising only 20-35 per cent. by dry weight of the total recovery. It was then found that the matter left behind in the centre cell was not really inactive, but gave vasoconstriction down to concentrations of 0·1 per cent., when an increase in capillary permeability made its appearance, first as a ring, then at 0·05 per cent. concentration as a homogeneous patch. If, as seems likely, the permeability-increasing activity of this fraction is masked by its vaso-constrictor effect, then the failure to demonstrate increased activity in the peptides migrating to the cathode becomes explicable. Whether the behaviour of the centre-cell fraction in the permeability test is a property of the peptides themselves or of impurities cannot be answered. The near anode cell contained merely traces of peptides, the far anode only inorganic matter ; neither was active in the permeability test and together they accounted for about 30 per cent. of the total recovery by dry weight.

Both cathode and centre cells contained leucocyte-attracting and endothelial-swelling activity, and the former seemed the more potent, although the accuracy of the method does not justify a more precise comparison. Both anode compartments were inert in this respect.

The results were similar with two cathode compartments and one anode cell, except that a small quantity of relatively inert peptides collected in the near cathode chamber. As the *p*H of the starting solution was raised, so the amount of peptide migrating to the cathode declined. In addition, the quantity passing to the anode increased, but not to the extent that might be expected.

*Ionophoresis*. Ionophoresis at *p*H 4 was tried but no separation of the active principle was achieved. With a current of 20 milliamps the activity in the permeability test was pooled about 1/3 of the way to the cathode electrode after 2 hours. Material was recovered from the glass wool by squeezing the latter out at the end of the experiment, the wool being divided into compartments by bars of agar jelly.

*Iso-electric point precipitation*. When an aqueous solution of the ammonium sulphate precipitate is gradually rendered acid, a precipitate forms which reaches its maximum at an iso-electric point of *p*H 3·8 : this comprises 1-2 per cent. *w/w* of the total material. This iso-electric precipitate, however, has the same order of activity in the permeability leucocyte-emigration and endothelial-swelling tests as the rest of the ammonium sulphate precipitate. It appears to be formed from the peptides precipitated by ammonium sulphate between 0 and 20 per cent. saturation when the latter are evaporated to dryness from aqueous solution.

*Ammonium sulphate supernatant*. Attempts at separating the active principle from the ammonium sulphate precipitate having proved unsuccessful, the corresponding supernatant was turned to. As has been said, it is inert in the permeability test, but when tested for activity in inducing polymorph emigration and endothelial swelling it proved to possess the same order of potency as the ammonium sulphate precipitate. Massing of leucocytes around blood vessels was produced at concentrations of 4 per cent., and less constant and striking emigration down to 0·2 per cent. Prior to testing, the material had been freed of all but traces of free amino acids by prolonged electrodialysis at an acid *p*H in the apparatus described. Under these conditions the amino acids and a proportion of the peptides migrate to the cathode, leaving the peptides under discussion behind in the centre cell.

### *The different peptide fractions and their average chain lengths*

Having found the peptides of the ammonium sulphate supernatant to possess only two of the three biological properties of the corresponding precipitate, it was decided to compare their chemical properties by determining the number of amino acids in the average peptide chain of the two fractions. Similar determinations were performed on the peptides precipitated at $p$H 3·8 and on those salted out with ammonium sulphate between 0 and 20 per cent. and between 40 and 50 per cent. saturation, since they all differ in appearance or chemical properties although not in biological activity. All these fractions were prepared as described above, the two last being re-precipitated at the appropriate saturation level.

The average chain length of the ammonium sulphate supernatant was five amino acids ; of the ammonium sulphate precipitate ten ; of the $p$H 3·8 precipitate fourteen ; of the 0-20 per cent. saturation precipitate fourteen and of the 40-50 per cent. saturation precipitate eight amino acids. The total nitrogen of all these fractions was of the order of 13 per cent. except for the ammonium sulphate supernatant, where it was 6 per cent.

### *Acid hydrolysates and amino-acid composition*

Acid hydrolysates of all the fractions mentioned were prepared and their amino-acid composition determined by paper partition chromatography. All contained tyrosine, phenylalanine and leucine, with or without isoleucine, valine, histidine, arginine, lysine, $\beta$-alanine, threonine, glycine, serine and aspartic and glutamic acids. In addition, all but the ammonium sulphate supernatant gave proline and amino butyric spots. All neutralised acid hydrolysates were completely devoid of activity in the capillary-permeability, leucocyte-emigration and endothelial-swelling tests.

### *Tryptic digests*

When the ammonium sulphate precipitate was digested with commercial trypsin and the resulting mixture subjected to paper-partition chromatography the following amino acids were found to be liberated :—tyrosine, phenylalanine and leucine, with or without isoleucine, valine, arginine, lysine, histidine, $\beta$-alanine, glycine, serine and glutamic acid.

In addition, the precipitate after digestion had lost its power to increase capillary permeability, as reported by Duthie and Chain, using crystalline trypsin. However, it retained its ability to attract leucocytes and to induce capillary endothelium to swell in the same concentrations as before.

Incubation with commercial trypsin of the ammonium sulphate supernatant yielded the same amino acids as the salted-out precipitate, except that arginine and lysine were not liberated although present in the acid hydrolysate of the same material. After digestion, this fraction retained its power of inducing leucocyte emigration and of causing vascular endothelium to swell ; its inability to produce increased capillary permeability remained unchanged.

### *Surface activity*

Surface activity, *i.e.* ability to lower surface tension, is a general property of higher peptides. It is possessed by all the fractions so far mentioned except the acid hydrolysates, where all peptide bonds had been ruptured. Thus the ammonium sulphate precipitate supernatants and their tryptic digests all show surface activity easily demonstrated by frothing on shaking.

In an attempt to obtain support for the hypothesis that leucocytic emigration was related to surface activity various other surface-active substances were tested. These included rat serum albumin and globulins, Teepol in concentrations from 20 to 0·1 per cent., glucose and sodium octanoate. Turpentine in concentrations from 25 to 0·2 per cent. was also tried, as a non-surface-active irritant. In the highest concentrations Teepol and turpentine both appeared to increase the number of polymorphs in the lumen of the skin vessels but no emigration or endothelial swelling was produced by them or by any of the other substances tested.

### *Effect of anti-histamine drugs*

Five mg. of Anthisan (May and Baker) was injected intramuscularly into 3 rats, and 30 minutes later a test of capillary-permeability action was carried out, using the original digest. The appearance of dye-staining was greatly delayed and the intensity finally reached but a fraction of that normally obtained. Nevertheless, a positive response could be detected in the highest dilution normally yielding one. In the rabbit, the capillary-permeability effect appeared to be completely inhibited. In contradistinction to this, previous dosage with Anthisan had no effect on polymorph migration or on endothelial swelling.

### *Other enzymic digests of proteins*

Peptic digestion of blood albumin yields a mixture having slightly less activity in the permeability to dye tests than the corresponding digest of fibrin. Like the latter, the active principle is quantitatively precipitated by 75 per cent. saturation with ammonium sulphate.

Peptic digestion of gelatine has been reported not to yield an active product. In fact, in the rat, slight permeability increase is effected by the crude digest at a dilution of 1 per cent. When the digest is brought to 75 per cent. saturation with ammonium sulphate a precipitate forms which contains all the permeability activity and has an end-point of 0·1 per cent. Commercial trypsin yields digests active in the permeability and leucocyte-attracting tests, both from blood fibrin and from albumin ; in both cases the active material is quantitatively precipitated from solution by 75 per cent. saturation with ammonium sulphate.

### *A crystalline higher peptide*

Mr N. M. Green of the department of chemical pathology kindly gave me a sample of crystalline beef-pancreas trypsin inhibitor, a peptide with a molecular weight of the order of 6000 according to Northrop. This material increased capillary permeability in the rat at concentrations down to 0·005 per cent. and induced leucocytic emigration and endothelial swelling at a concentration of 0·4 per cent. Trypsin inhibitor is quantitatively precipitated by 75 per cent. saturation with ammonium sulphate.

### *Inflammatory exudates*

These were produced experimentally by injecting 1·5 ml. of turpentine into the pleural cavity of the goat, 0·2 ml. of turpentine into the pleural cavity of the rabbit and 50 ml. of sterile 0·9 per cent. saline into the peritoneal cavity of the guinea-pig. In all cases the animals were killed 24 hours later, the exudate collected, freed from cells and deproteinised with acetone and pyridine as described for protein digests by Cullumbine and Rydon.

The rabbit turpentine exudate contained free tyrosine and leucine, with or without isoleucine, valine, proline, arginine and glycine as demonstrated by paper partition chromatography. It possessed permeability-increasing and leucocyte-attracting properties and on bringing to 50 per cent. saturation with ammonium sulphate yielded a precipitate which contained all the permeability and leucocyte-attracting activity.

The guinea-pig saline exudate contained free tyrosine, phenylalanine and leucine, with or without isoleucine and valine as demonstrated by paper partition chromatography. It possessed weak capillary-permeability-increasing activity and also the power of inducing leucocyte emigration. Ammonium sulphate fractionation was not performed.

From the goat, 70 ml. of turbid fluid containing large numbers of leucocytes were obtained yielding 80 mg. of precipitate on bringing to 75 per cent. saturation with ammonium sulphate after deproteinisation. This material was partially soluble in water and the solution or suspension was surface active, as were the other two exudates described above. The ammonium sulphate supernatant showed no surface activity. On paper partition chromatography the precipitate was seen to contain no free amino acids. On acid hydrolysis, it yielded tyrosine, phenylalanine and leucine, with or without isoleucine, valine, histidine, glycine, serine, glutamic acid and aspartic acid; it was found to possess five amino acids per average peptide chain.

The ammonium sulphate precipitate induced increased capillary permeability at dilutions down to 1 per cent., and polymorph emigration and endothelial swelling down to 2 per cent.

### *Experimental burns*

Anæsthetised rats were shaved and a test-tube containing water at 80° C. was held in contact with the abdominal skin for 90 seconds and the time of appearance of increased permeability to trypan blue, previously injected intravenously, determined. In other rats skin sections were taken at various intervals and leucocyte emigration and endothelial swelling looked for.

Increased permeability appeared immediately and reached its maximum within 5 mins., the dye diffusing out widely from the burnt area. Leucocyte emigration and endothelial swelling did not appear until half to one hour later. At half an hour blood stasis seemed to have begun, judging from the fixed sections. Macroscopic evidence of accumulation of fluid in the skin was not apparent for an hour.

## DISCUSSION

That inflammation is accompanied by evidence of tissue breakdown has long been known, but until Menkin was able to reproduce some of the changes of acute inflammation with cell-free preparations of inflammatory exudates obtained from the pleural cavities of dogs by the injection of turpentine its possible significance was not realised (Opie, 1922).

Menkin (1936), after observing that the active principle was precipitated with the albumin fraction of the exudate by ammonium sulphate and that it was dialysable, went on to devise a scheme for its extraction (Menkin, 1938). This involved extraction with pyridine and acetone followed either by extraction with butanol or separation of the active principle from the acetone supernatant at —20° C. In both cases the product was claimed to be crystalline although not to be pure. In 1939 Duthie and Chain had the opportunity of examining a specimen of Menkin's product, which he had dubbed " leucotaxine ", and reported it to be amorphous, although otherwise as the author had described it.

Apart from some possible removal of fatty material by the solvent pyridine, the acetone/pyridine extraction was in fact simply a deproteinisation, the extract containing several free amino acids and large quantities of inorganic and probably of carbohydrate material besides the active principle. Since the latter comprises material both soluble and insoluble in water the ability of Menkin to produce both butanol- and water-soluble fractions can be understood, as the water-insoluble active material can be extracted by prolonged shaking with organic solvents including butanol. Duthie and Chain suggested that this property of solubility in butyl alcohol was imparted by combination of the active material with lipoids and the results of chromatography, particularly on the alcohol-soluble fractions of the peptic digest of fibrin, support this.

### *A crystalline higher peptide*

Mr N. M. Green of the department of chemical pathology kindly gave me a sample of crystalline beef-pancreas trypsin inhibitor, a peptide with a molecular weight of the order of 6000 according to Northrop. This material increased capillary permeability in the rat at concentrations down to 0·005 per cent. and induced leucocytic emigration and endothelial swelling at a concentration of 0·4 per cent. Trypsin inhibitor is quantitatively precipitated by 75 per cent. saturation with ammonium sulphate.

### *Inflammatory exudates*

These were produced experimentally by injecting 1·5 ml. of turpentine into the pleural cavity of the goat, 0·2 ml. of turpentine into the pleural cavity of the rabbit and 50 ml. of sterile 0·9 per cent. saline into the peritoneal cavity of the guinea-pig. In all cases the animals were killed 24 hours later, the exudate collected, freed from cells and deproteinised with acetone and pyridine as described for protein digests by Cullumbine and Rydon.

The rabbit turpentine exudate contained free tyrosine and leucine, with or without isoleucine, valine, proline, arginine and glycine as demonstrated by paper partition chromatography. It possessed permeability-increasing and leucocyte-attracting properties and on bringing to 50 per cent. saturation with ammonium sulphate yielded a precipitate which contained all the permeability and leucocyte-attracting activity.

The guinea-pig saline exudate contained free tyrosine, phenylalanine and leucine, with or without isoleucine and valine as demonstrated by paper partition chromatography. It possessed weak capillary-permeability-increasing activity and also the power of inducing leucocyte emigration. Ammonium sulphate fractionation was not performed.

From the goat, 70 ml. of turbid fluid containing large numbers of leucocytes were obtained yielding 80 mg. of precipitate on bringing to 75 per cent. saturation with ammonium sulphate after deproteinisation. This material was partially soluble in water and the solution or suspension was surface active, as were the other two exudates described above. The ammonium sulphate supernatant showed no surface activity. On paper partition chromatography the precipitate was seen to contain no free amino acids. On acid hydrolysis, it yielded tyrosine, phenylalanine and leucine, with or without isoleucine, valine, histidine, glycine, serine, glutamic acid and aspartic acid ; it was found to possess five amino acids per average peptide chain.

The ammonium sulphate precipitate induced increased capillary permeability at dilutions down to 1 per cent., and polymorph emigration and endothelial swelling down to 2 per cent.

a peptide fraction has been obtained containing an average of five amino acids per chain which can cause leucocytes to emigrate from blood vessels and vascular endothelium to swell with no corresponding effect on vessel permeability to dye particles. For these reasons the conception of "leucotaxine" as a single entity can no longer be entertained. The sum-total of the results of ammonium sulphate fractionation is to suggest that, from 8 amino acids per chain upwards, peptides produce changes in vessel permeability, leucocyte emigration and endothelial swelling; while with five amino-acid residues or less, only the last two effects can be achieved. It also seems that the largest peptides, with 14 amino-acid residues per chain, can acquire the property of being quantitatively precipitated at an iso-electric point of $p$H 3·8 by evaporation to dryness, with no corresponding change in their biological properties. These findings cannot, of course, be extended beyond the peptic digest of fibrin.

Further evidence for the biological actions described being the property not of one, but of a wide group of peptides, is provided by the results of the experiments with trypsin, where it was shown that the same enzyme that liberated peptides active in the permeability test from proteins destroyed the permeability effect of peptides formed by the action of pepsin on these same proteins. This indicates that structural as well as chain-length differences are compatible with the retention of the biological properties under discussion.

Further support for this hypothesis is provided by the results with crystalline trypsin inhibitor. This is a polypeptide, said by Northrop to have a molecular weight around 6000. It is of interest that, as well as having similar effects on vessel permeability and leucocytes, it has the same order of activity as the peptide mixture salted-out from the peptic digest of fibrin.

That permeability-increasing activity can be destroyed by trypsin while leaving leucocyte-attracting powers unchanged is a finding which tallies with the biological effects of the ammonium sulphate supernatant of the peptic digest of fibrin, since the products of tryptic digestion are probably of the same order of chain length as the peptides in this fraction. On the other hand, that peptides having all three biological properties have something in common is suggested by the quantitative precipitation by 50-75 per cent. saturation with ammonium sulphate, which all such peptides so far studied have shown. This statement is true of peptic and tryptic digest of fibrin, albumin and gelatin and also of inflammatory exudate. The most likely form for this similarity to take is one of chain length or molecular size; quantitative differences such as exist between fibrin and gelatine peptic digests could be due to variations either in amino-acid composition, related to that of its parent protein, or to differences in the number of peptides of the appropriate chain length produced.

The peptide nature of all the active principles seems now to be established. They are liberated from proteins by proteolytic enzymes;

the active principle occupies the area on chromatograms taken up by higher peptides ; total disruption of peptide bonds by acid hydrolysis totally destroys activity ; partial disruption of peptide bonds by commercial preparations of trypsin destroys the activity believed to be related to the larger fragments, *i.e.* the permeability effect ; the active principles are soluble in the protein solvents, can be salted out between 10 and 75 per cent. saturation, can acquire the property of being precipitated at an iso-electric point, have been shown to be amphoteric by electrodialysis at varying *p*H levels, and are surface-active. Finally, a crystalline higher peptide, trypsin inhibitor, has been found to possess the biological properties under discussion.

The active principle of inflammatory exudate possesses enough of these properties to make its peptide nature seem beyond doubt. Its formation is accompanied by definite evidence of protein breakdown, *i.e.* free amino acids are present in the exudate ; it is quantitatively removed by salting-out ; destruction of the peptide linkage by acid hydrolysis destroys its activity and at the same time liberates its constituent amino acids ; and it is surface-active.

The dissociation of permeability effect and leucocytic and endothelial effects has been achieved by ammonium sulphate fractionation, by tryptic digestion, and by means of Anthisan. This dissociation seems to rule out the possibility that the leucocyte emigration is a side effect of the increase in the permeability of the vessel wall to particles such as dye.

Although no preparation has been obtained able to attract polymorphs which was not also surface-active, it has not been found possible to destroy the latter property without also destroying all the peptide links and reducing the substance to its constituent and inert amino acids. Nor is the failure of other powerful surface-active agents to induce leucocyte emigration conclusive one way or the other. For want of definite evidence to the contrary it seems reasonable to suppose that peptides attract polymorphs from the circulation by virtue of a specific power of chemotaxis.

### *The role of peptides in inflammation*

In the experimental burning of rats' skin described, the increase in capillary permeability was immediate and extensive. The speed with which it appeared seems to rule out the possibility that peptides produced by tissue destruction could be responsible. These peptides as far as we know could only be formed by the action of proteolytic enzymes, liberated from cells or activated in serum, on cellular and extracellular protein perhaps previously denatured by the initial insult. Such a reaction could not proceed rapidly enough to yield peptides in quantity sufficiently large to effect an intense and immediate permeability increase.

The perpetuation of increased vessel permeability, as seen by the gradual accumulation of œdema fluid at the experimental burn site and the delayed appearance of polymorph emigration and endothelial swelling there, are compatible with the formation and gradual accumulation of peptides in the tissues. Experimental burns of the rabbit's ear followed by means of the Clark's chamber technique do not yield real masses of polymorphs until some hours have elapsed (Bangham, unpublished observation). Clearly the accumulation of leucocytes in burned tissue is related to the gradual formation of some substance from the tissues themselves. There is no evidence that the products of fat and carbohydrate breakdown are active in this respect, nor is inorganic material. Of protein breakdown products, only peptides between certain size limits have been found to reproduce the signs of inflammation. Both cells and serum contain proteolytic enzymes of the type able to yield these peptides. It seems reasonable to conclude that, at least in burning, the changes of inflammation are partly related to the formation of peptides at the site of injury.

The inflammatory exudate produced in the goat's pleural space by turpentine is rich in polymorphs and exuded serum and contains a concentration of peptides of the order of 0·1 per cent. This level is adequate to maintain increased vessel permeability and to induce leucocyte emigration. Moreover, the concentration in the tissues whence the polymorphs are derived, *i.e.* in relation to the small vessels of the pleura and lung, is probably a good deal higher. The peptides present are of the order of chain length able to attract leucocytes while, if the analogy of peptic digests of fibrin is applicable, their relatively weak permeability effect can be due to the presence of some longer peptide chains in the precipitate analysed, which is almost certainly a mixture of such peptides of different sizes.

It seems that much evidence exists for regarding higher peptides as involved in producing some of the changes of acute inflammation. Although only the simple cases of burn and chemical inflammation have been considered in this paper, it is difficult to see how bacterial invasion could fail to contribute to the local concentration of peptides by virtue of the proteolytic enzymes and substrate protein of the organisms themselves.

## Summary

1. The ability of certain enzymic digests of proteins to reproduce some of the features of acute inflammation, namely to increase capillary permeability, to induce polymorph emigration from the vessels and to cause vascular endothelium to swell, was investigated.

2. The active principle of all such digests was found to be the peptide fraction, and in every case to be salted out by 75 per cent. saturation with ammonium sulphate and to show surface activity.

3. In the case of a peptic digest of fibrin the biological properties described were found to be shared by peptides with an average chain length of from 14 to 8 amino-acid residues. From the same digest

a peptide fraction was obtained which was able to induce polymorph emigration and endothelial swelling but had no effect on vessel permeability and whose average peptide chain contained only five amino acids.

4. A pure crystalline peptide, pancreatic trypsin inhibitor, with an estimated molecular weight of 6000, was found to possess all three of the biological properties under discussion.

5. For these and other reasons it is concluded that such properties are possessed not by one but by many peptides within certain limits of molecular size, and that the biological effect may vary with the length of the peptide chain.

6. Experimentally-induced inflammatory exudate, in conformity with previous workers, was found to possess leucocyte-attracting and endothelial-swelling powers, and relatively weak permeability-increasing activity. In the case of turpentine-induced goat pleural exudate, the active principle of all three properties was the peptide fraction, and probably a mixture of peptides whose average chain length was five amino acids.

7. It is concluded that much evidence exists for peptides of the size discussed in this paper being partly responsible, as a result of their liberation at sites of tissue injury, for some of the changes of acute inflammation.

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# EFFECTS OF ANTERIOR HYPOPHYSIS HORMONES, ALONE AND WITH SOME OTHER HORMONES, ON AGEING FEMALE RATS

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(Plates XII-XVI)

The literature on the hormones of the anterior lobe of the hypophysis (abbreviated in the text as A.H. hormones) is very extensive. It has been well reviewed in the books by Burrows (1945, and 2nd ed., 1949) and Selye (1947). In this paper it is only possible to refer to some articles closely related to the results of our experiments on female rats.

In the present experiments, the relationship is studied between the A.H. hormones and androsterone (male hormone naturally present in the female organism), progesterone, desoxycorticosterone acetate (referred to in the text as desoxycorticosterone) and thyroid hormone.

An investigation of the simultaneous effects of different hormones is important for various reasons. (1) In the organism, all hormones are secreted simultaneously. (2) Some hormones become effective or their unfavourable effects are neutralised only in co-operation with certain other hormones. (3) Unless there is definite evidence of a single deficiency, therapeutic treatment with one hormone only might produce artificial hypersecretion of the respective hormone and so cause a dangerous upset in the hormonal balance.

Experiments were performed on 80 normal female rats aged about 17 months. At this age rats have already started to show symptoms of ageing. For example, they begin to lose the smoothness and lustre of the fur, they show a considerable degree of relative hypoplasia in some organs (Korenchevsky, 1942 ; Korenchevsky and Jones, 1946) and they become less agile.

## TECHNIQUE

The duration of the experiment was 42 days. A.H. extract from bovine pituitaries, prepared in the form of a powder by Messrs Organon Ltd., was used. A considerable difficulty encountered in experiments with A.H. hormones is the development in some cases of anti hormones which neutralise the specific effects. From this point of view, administration of different doses is helpful in the interpretation of the results. In the present investigation two doses of A.H. extract, 3 and 6 mg., were given four times a week. Progesterone (2 mg. four times a week) was injected as an oily solution. Desoxycorticosterone was implanted subcutaneously in the form of sterile 25 mg. pellets prepared by Messrs Organon Ltd. Desiccated thyroid was given as a sweetened gum-acacia suspension by pipette in doses of 65 mg. three times a week. The number and distribution of rats in the various groups is given in table I.

The renal convoluted tubules were measured as paper replicas by the method previously described (Korenchevsky and Hall, 1944).

The organs were fixed in Bouin's fluid, embedded in paraffin and stained with iron hæmatoxylin and eosin. For fatty substances frozen sections were stained with sudan black.

All results were statistically analysed by Mr J. L. Nicholson of the Institute of Statistics, Oxford University, to whom we express our gratitude. The statistical results are expressed in P (probability) values. When two P values are given (*e.g.* $P<0{\cdot}05$-$0{\cdot}01$) they varied in the different groups referred to, but lay on any figure between the limits quoted.

## EXPERIMENTAL FINDINGS

### *Changes in body weight and fat deposition*

A statistically significant increase in body weight was obtained only with combinations containing androsterone (table I, cols. 7, 8 and 9; $P<0{\cdot}05$-$0{\cdot}01$). This result confirms previous observations (Korenchevsky, Dennison and Levy Simpson, 1935; Korenchevsky, Hall and Burbank, 1939; Korenchevsky and Jones, 1946).

In spite of the absence of any significant changes in body-weight, a definite decrease of fat deposition (as judged by the amount of abdominal fat) was found in the groups treated simultaneously with A.H. extract, progesterone and thyroid hormone (cols. 5 and 11; $P<0{\cdot}02$-$0{\cdot}01$), or with A.H. extract and desoxycorticosterone (col. 7; $P<0{\cdot}05$). This, in agreement with our previous observations, indicates that, whereas the metabolism of fat tissue was increased, in other tissues and organs the catabolic processes predominated over the anabolic.

### *Changes in the ovaries*

The effects of the anterior hypophysis on the ovaries are due to three hormones :—(1) follicle-stimulating (FSH), (2) luteinising (LH, also defined as interstitial-cell-stimulating) and (3) luteotropic (LTH, also called prolactin). They produce respectively (1) development of follicles, (2) formation of corpora lutea and stimulation of interstitial cells and (3) preservation of corpora lutea, stimulation of their secretion (progesterone) and, in the mammary glands, milk secretion.

Excessive doses of the A.H. hormones might produce pathological lesion in the ovaries, namely ovarian cysts (Burrows, 1945, p. 25), or atrophy of the follicles and corpora lutea with subsequent extensive development of interstitial tissue cells (Desclin, 1949). Thyroid hormone increased the weight of the ovaries in experiments by Hayashi (1929), Korenchevsky, Hall and Clapham (1943) and Korenchevsky, Paris and Benjamin (1950). In such ovaries Weichert

## TABLE I

*Effects of androgenic, thyroid and anterior hypophysis hormones, progesterone and desoxycorticosterone acetate on weights of organs of ageing female rats*

| Organs | Controls | Progesterone + desiccated thyroid | Anterior lobe extract 3 mg | | | | | | Desoxycorticosterone + androsterone | Anterior lobe extract 6 mg | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Alone | + desiccated thyroid | + progesterone + desiccated thyroid | + desoxycorticosterone | + androsterone | + desoxycortic + androsterone | | Alone | + progesterone + desiccated thyroid |
| Abdominal fat (g) | 10.4 | 8.3 | 8.2 | 7.4 | 5.7 | 6.5 | 11.7 | 9.1 | 12.6 | 9.2 | 4.6 |
| Ovaries (mg) | 64 | 72 | 85 | 98 | 78 | 74 | 69 | 76 | 124 | 64 | 74 |
| Uterus (mg) | 422 | 357 | 380 | 358 | 281 | 403 | 352 | 419 | 396 | 391 | 284 |
| Vagina (mg) | 390 | 447 | 425 | 421 | 435 | 455 | 434 | 407 | 438 | 449 | 398 |
| Preputial glands (mg) | 108 | 130 | 142 | 144 | 178 | 123 | 225 | 240 | 199 | 151 | 185 |
| Adrenals (mg) | 60 | 68 | 65 | 73 | 74 | 67 | 45 | 46 | 46 | 68 | 78 |
| Thyroid (mg) | 16.5 | 19.1 | 18.0 | 18.3 | 18.0 | 17.7 | 18.1 | 17.7 | 19.0 | 17.5 | 17.4 |
| Hypophysis (mg) | 14.0 | 12.5 | 13.7 | 11.8 | 13.0 | 11.9 | 12.0 | 11.4 | 12.1 | 14.4 | 13.4 |
| Thymus (mg) | 106 | 134 | 98 | 172 | 153 | 122 | 121 | 142 | 105 | 107 | 163 |
| Liver (g) | 7.84 | 8.31 | 7.96 | 9.79 | 9.74 | 8.66 | 8.16 | 8.79 | 8.26 | 9.39 | 10.00 |
| Kidneys (g) | 1.50 | 1.64 | 1.54 | 1.75 | 1.73 | 1.57 | 1.51 | 1.70 | 1.71 | 1.57 | 1.79 |
| Spleen (mg) | 644 | 755 | 681 | 935 | 1020 | 784 | 651 | 759 | 802 | 817 | 1099 |
| Heart (mg) | 770 | 890 | 811 | 945 | 965 | 870 | 823 | 926 | 846 | 895 | 965 |
| Brain (mg) | 1501 | 1498 | 1427 | 1487 | 1435 | 1479 | 1429 | 1406 | 1464 | 1469 | 1454 |
| Final body wt (g) | 220 | 228 | 224 | 233 | 232 | 230 | 234 | 234 | 235 | 233 | 231 |
| Gain in body wt (g) | 12 | 13 | 8 | 17 | 0 | 20 | 23 | 25 | 28 | 10 | 21 |
| No of rats in group | 8 | 7 | 7 | 7 | 7 | 7 | 8 | 9 | 7 | 6 | 7 |
| Columns | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |

and Boyd (1933-34) recorded development chiefly of the corpora lutea, while in the experiments of Korenchevsky, Paris and Benjamin (1950) small doses of thyroid hormone increased the number of corpora lutea or follicles or both. Fluhmann (1934) and Tyndale and Levin (1937), using apparently toxic doses of thyroid hormone, found a decrease in the weight of the ovaries. A depressing effect of toxic doses is typical for this hormone.

Large doses of progesterone and desoxycorticosterone acetate, according to Selye and Friedman (1940), produce ovaries that are atrophic or of about normal size. They have atretic follicles, but may contain some enlarged corpora lutea.

In the present experiments with A.H. extract, ovarian hypertrophy was obtained (table I). It was, however, statistically significant only with a large dose ($P<0{\cdot}001$). The "not significant" effect of a small dose of A.H. extract became significant ($P<0{\cdot}02$) when thyroid hormone was administered simultaneously (table I, col. 4). This cooperative stimulating effect was counteracted by the simultaneous administration of progesterone (table I, cols. 5 and 11). An apparent increase in weight of the ovaries observed in some other groups was not statistically significant.

Histologically in most of our control rats fully developed ovaries were observed, with mature follicles and corpora lutea, and numerous cells of the interstitial gland well loaded with lipoids (fig. 1). In one rat only, premature ageing of the ovaries was observed: most of the follicles and corpora lutea were in various stages of atresia, which is typical for ovaries of older (senescent) rats.

In the treated groups, various degrees of depressed development of the follicles and stimulation of the corpora lutea were present in all rats which received A.H. extract, alone or in combination with other hormones, androsterone excepted. Depression of the follicles was expressed by a decrease in their number and size, immature forms of various grades being present. Stimulation of the corpora lutea resulted chiefly in an increase in their number, more rarely in size (figs. 2, 3, 5 and 6).

In the rats injected with both androsterone and A.H. extract, not only was the development of the follicles depressed (fig. 6), but, exceptionally, that of the corpora lutea as well (fig. 4). In such cases general atrophy of the ovaries was present. This effect should be explained by the well-established depressing action of androgens on the gonadotropic secretion of the hypophysis, and, through this, on the ovaries. Even the average weight of the ovaries in these "androgenic" groups showed that in most rats such atrophying action of androgens was prevented by A.H. hormones. Fig. 3 shows that the stimulating effect of the A.H. hormones could not only neutralise but might even predominate over the depressing androgenic effect. No definite effect of desoxycorticosterone was observed in the ovaries.

Comparison between figs. 2 and 5 shows that progesterone lessens the hypertrophying effect of A.H. and thyroid hormones; the corpora lutea become less numerous and most of them smaller.

Thus the main results obtained can be summarised in three

## PLATE XII

Hæmatoxylin and eosin. ×12

FIG. 1.—Control rat. Normal structure of ovary, containing both follicles and corpora lutea. Wt. of ovaries 77 mg.

FIG. 2.—Rat treated with A.H. extract (3 mg.) and thyroid hormone. Better developed ovary, with more numerous and some larger corpora lutea. The most nearly normal structure as compared with that in figs. 1 and 3-6. Wt. of ovaries 131 mg.

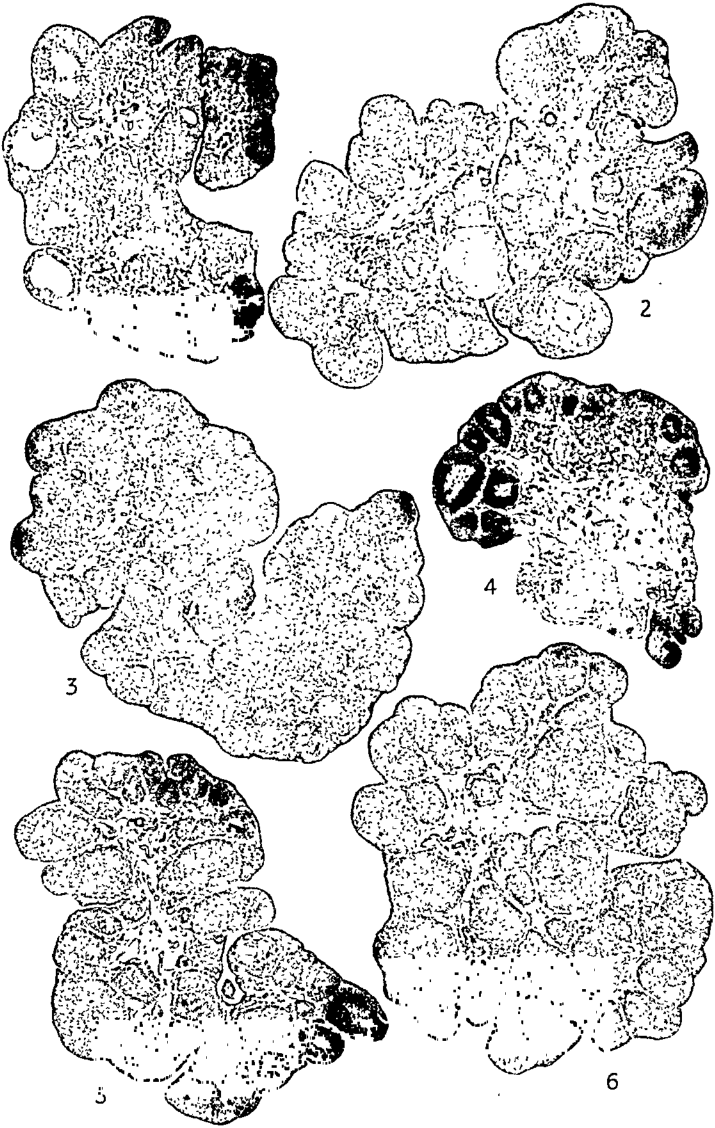
FIG. 3.—Rat treated with A.H. extract (3 mg.) and androsterone. The atrophying "androgenic" effect has been counteracted by the hypertrophying action of A.H. hormones. Wt. of ovaries 110 mg.

FIG. 4.—Rat treated with A.H. extract (3 mg.), androsterone and desoxycorticosterone acetate. Atrophic ovary with considerable increase of fibrous tissue. In this case A.H. hormones failed to neutralise the atrophying effect of androsterone. Wt. of ovaries 56 mg.

FIG. 5.—Rat treated with A.H. extract (6 mg.), progesterone and thyroid hormone. A smaller and histologically less well-developed ovary than in fig. 2, consisting almost entirely of corpora lutea. Wt. of ovaries 46 mg.

FIG. 6.—Rat treated with a large dose of A.H. extract (6 mg.). Hypertrophic ovary, consisting almost exclusively of corpora lutea. Wt. of ovaries 146 mg.

OVARIES IN TRANSVERSE SECTION



conclusions :—(1) The A.H. extract used contained chiefly luteinising and luteotropic hormones, but not the follicle-stimulating one; (2) it was able to neutralise and in some cases even to predominate over the depressing effect of androgens on the ovaries; (3) a synergistic relationship was observed between A.H. hormones and thyroid hormone.

### *Changes in the uterus*

A definite change—a decrease in the weight of the uterus—was observed only in the groups treated simultaneously with progesterone, thyroid hormone and A.H. extract, in both small ($P<0{\cdot}01$) and large ($P<0{\cdot}001$) doses (table I, cols. 5 and 11).

Histologically, with A.H. extract alone, at both dosage levels, the uterus was in the diœstrus condition in all rats except one, which showed the progestational structure.

Various degrees of progestational changes were found in most rats receiving progesterone in combinations with other hormones. Comparison of the group "progesterone+thyroid hormone" with the group "progesterone+thyroid hormone+A.H. extract" showed considerably more pronounced progestational changes with the latter combination of three hormones. This indicates that A.H. hormones increased the progestational effect of progesterone on the uterus.

In all androsterone combinations diœstrus was the usual condition of the uterus. In previous experiments it was found that androsterone, by depressing the gonadotropic function of the hypophysis, produces a decrease in the size and weight of the uterus (Korenchevsky, Paris and Benjamin). In the present experiments this androgenic effect was not significant in any combination with A.H. extract (table I, cols. 7 and 8) and desoxycorticosterone (col. 8), since these hormones apparently neutralise the depressing action of androsterone. As in previous experiments (Korenchevsky, Hall and Clapham), no definite effect of thyroid hormone on the uterus of normal rats was observed. Previously, however, its slightly stimulating action on the uterine epithelium and myometrium in ovariectomised rats was recorded (Korenchevsky and Jones, 1947).

In transverse sections of the uterine horn, in the region of greatest diameter, the thickness of the mucosa and myometrium was measured by ocular micrometer and their ratio ascertained in all rats in the present experiment. This ratio in control rats was, on the average, 2·22. It was only slightly less (1·8-2·04) in all rats receiving androsterone or desoxycorticosterone in the combinations used.

In the animals receiving A.H. extract, alone or with thyroid hormone or progesterone or both, and also with the combination of progesterone and thyroid hormone only, this ratio was definitely smaller, ranging from 1·33 to 1·65. Therefore with all these latter combinations the development of the myometrium was relatively more pronounced than that of the mucosa.

### *Changes in the vagina*

In previous experiments androsterone was found to have a hypertrophying effect on the vagina in ovariectomised rats (Korenchevsky, Hall and Burbank; Korenchevsky and Jones), but not in normal intact rats (Korenchevsky, Paris and Benjamin).

Weichert and Boyd produced progestational mucification in the vagina of rats treated with large doses of thyroid hormone. Korenchevsky, Hall and Clapham, however, observed the usual cyclic changes in the vagina, ranging from diœstrus to œstrus, and only exceptionally—in 2 out of 34 rats—the first incomplete stages of mucification.

In the present experiments, although in almost all the treated rats the weight of the vagina was greater than in the controls, it was statistically significant in one group only, namely that treated simultaneously with a small dose of A.H. extract, androsterone and desoxycorticosterone (table I, col. 8; $P<0{\cdot}02$). With A.H. extract alone, about half the treated rats showed varying degrees of progestational mucification of the vaginal epithelium; in the remainder diœstrus was present. It is noteworthy that, in spite of the luteinising effect of A.H. extract on the ovaries, progestational changes in the uterus were present in 2 out of 13 rats and in the vagina in about half of the animals only. In all rats, however, to which progesterone was given in various combinations, vaginal mucification was found, confirming previous results. While the addition of desoxycorticosterone to the other hormones injected had no definite effect, androsterone intensified the vaginal mucification in combination with the A.H. extract.

### *Changes in the female preputial glands*

The hypertrophying effect of androgens on the preputial glands has been described by several workers, confirming the results obtained by Korenchevsky, Dennison and Levy Simpson, and Korenchevsky, Hall and Burbank. It has also been recorded after injection of progesterone (Selye, 1940*a*) and A.H extract (Selye and Clarke, 1943). Hypertrophy of these glands was often observed in rats after the administration of thyroid hormone, but, because of its inconsistency, was not statistically significant (Korenchevsky, Hall and Clapham; Korenchevsky, Paris and Benjamin).

In the present experiment the weight of the preputial glands was greater than in the controls in all the treated groups, but statistically significant hypertrophy was only found with combinations containing androsterone ($P<0.001$), and with A.H. extract+thyroid hormone+progesterone ($P<0{\cdot}01$-$0{\cdot}001$). Since these latter hormones had no significant effect separately, a synergistic effect on the preputial glands is indicated.

### *Changes in the adrenals*

Atrophy of the adrenals after the injection of androgens has been repeatedly observed in spayed rats (*Korenchevsky, Dennison and Levy Simpson*, 1935; Korenchevsky, Hall and Burbank, 1939; Korenchevsky and Hall, 1940; Korenchevsky and Jones, 1946), but in normal rats, only on prolonged administration (Korenchevsky, Paris and Benjamin, 1950); it was not found after

## PLATE XIII

Outer part of cortex, with (from top to bottom of photographs) fibrous capsule, zona glomerulosa, demarcation zone and upper part of zona fasciculata. Hæmatoxylin and eosin. ×225.

FIG. 7.—Control rat. Normal structure. Adrenals 55 mg.

FIG. 8.—Rat treated with androsterone and desoxycorticosterone acetate. Depletion of lipoids in narrower zona glomerulosa, widened demarcation zone and decrease in size of cells and some nuclei in zona fasciculata. Adrenals 43 mg.

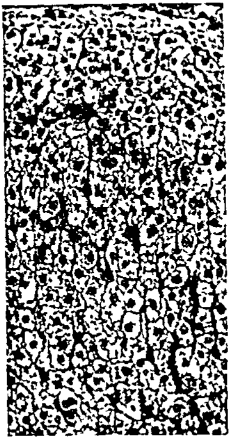
FIG. 9.—Rat treated with A.H. extract (3 mg.) and androsterone. Changes similar to those in fig. 8, but demarcation zone is wider. Adrenals 55 mg.

FIG. 10.—Rat injected with a large dose of A.H. extract alone. Structure of adrenal similar to that of control rats, but cells of zona fasciculata smaller. Adrenals 55 mg.

FIG. 11.—Rat treated with A.H. extract (3 mg.), progesterone and thyroid hormone. Hypertrophied adrenal, but structure similar to that in fig. 10. Adrenals 90 mg.

FIG. 12.—Rat treated with A.H. extract (3 mg.) and thyroid hormone. Adrenal enlarged, but histologically similar to controls. Adrenals 68 mg.

# ADRENALS



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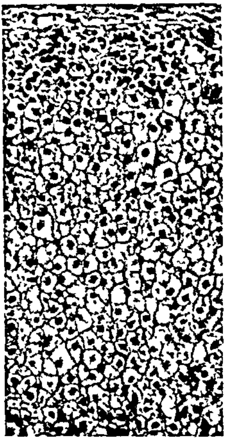

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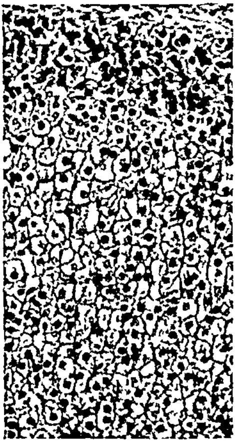

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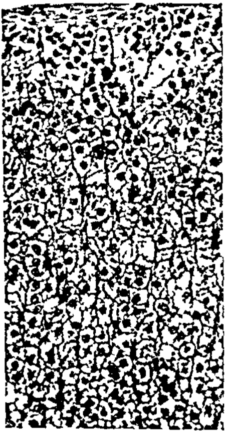

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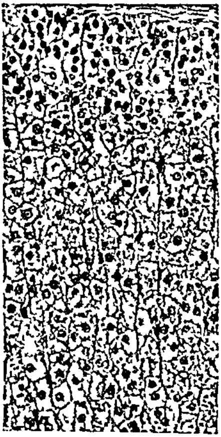

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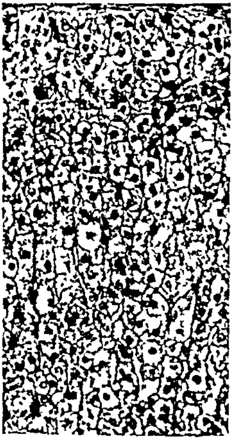

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a short treatment of, for example, three weeks' duration (Korenchevsky, Dennison and Hall, 1937*a* and *b*). Atrophy of these glands has also been recorded after administration of very large doses of progesterone and desoxycorticosterone acetate by Selye (1940*b*), but not by Forster *et al.* (1946), while Kimeldorf and Soderwall (1947) observed hypertrophy after the administration of moderate amounts of progesterone.

Hypertrophy of the adrenals was obtained after the treatment of rats with thyroid hormone (Hoskins, 1916; Cameron and Carmichael, 1921; Korenchevsky, Hall and Clapham, 1943; Korenchevsky and Jones, 1946, 1947; Korenchevsky, Paris and Benjamin, 1950). It is the cortex only which shows these atrophic or hypertrophic changes (Korenchevsky and Jones, 1947; Korenchevsky, Paris and Benjamin, 1950).

The presence of a corticotropic hormone in the anterior hypophysis and its extracts has now been well established and the substance has been isolated in pure form. Provided there is no counteraction by anti-hormone, corticotropic hormone in A.H. extracts produces hypertrophic changes in the adrenals (Selye and Clarke, 1943; Bergner and Deane, 1948; Dontigny *et al.*, 1948).

In the present experiments, A.H. extract alone, at both dosage levels, produced only statistically non-significant enlargement of the adrenals (table I). Histologically, however, the cortical cells were smaller (fig. 10) than in the control rats (fig. 7). Since the weight of the adrenals was not reduced, the increased number of cells should be explained as a hyperplasia produced by the adrenotropic hormone present in the A.H. extract used.

In combination with thyroid hormone (table I, cols. 4, 5 and 11) a statistically significant hypertrophy of the adrenals was obtained ($P<0{\cdot}01$-$0{\cdot}001$). It had previously been found (Korenchevsky and Jones, 1946) that, in the small doses used in this experiment, thyroid hormone alone did not cause significant hypertrophy of the adrenals. There is therefore evidence of a synergistic action of thyroid hormone and A.H. hormones on the adrenals. Histologically (fig. 12), although the cortical cells of these adrenals are larger than those with A.H. extract alone, they do not approach the size of the cells in the control rats. With these combinations of hormones also, therefore, hyperplasia and not hypertrophy of the cortical cells was the cause of the adrenal enlargement.

The depressing effect of desoxycorticosterone, observed by Selye, was not found in the present experiments. The depressing effect of androgens on the adrenals, previously observed by us, was again found in the present experiments ($P<0{\cdot}001$; table I, cols. 7, 8 and 9). The simultaneous administration of A.H. hormones or desoxycorticosterone did not neutralise this effect (figs. 8 and 9). Depletion of lipoid granules in the zona glomerulosa and widening of the demarcation zone were observed in the adrenals of the rats treated with hormonal combinations containing androsterone (figs. 8 and 9).

### *Changes in the thyroid*

The presence of thyrotropic hormone in the anterior hypophysis is well established. In A.H. extracts it is responsible for the stimulation of the growth and hormonal secretion of the thyroid gland. In previous papers (Korenchevsky, Hall and Clapham, 1943; Korenchevsky and Jones, 1946, 1947, 1948) the literature referring to the action of androgens, progesterone and thyroid hormone on the thyroid gland and the results of their own experiments on the subject have been given. It was found that androgens and progesterone stimulate, while thyroid hormone depresses the thyroid gland.

In the present experiments an increase in the weight of the thyroid was observed in all the treated groups, but this did not attain statistical significance. Histologically, in control rats, a slightly active condition of the thyroid was observed, with cubical or slightly higher epithelium and for the most part with a medium amount of colloid (fig. 13). The depressing effect of thyroid hormone could not be neutralised by progesterone (fig. 14).

The stimulating effect of A.H. extract was evident only with large doses, which considerably reduced the amount of colloid in the follicles and also neutralised the depressing effect of the thyroid hormone (fig. 16). In 4 out of 7 rats receiving all three hormones, the thyroid gland was even more active (fig. 16) than in the control rats (fig. 14). This demonstrates the overwhelmingly powerful effect of thyrotropic hormone in relation to the effect of the thyroid hormone (in certain doses) on this gland. The stimulating effect of androsterone on the thyroid was observed in most rats treated with combinations containing this hormone (fig. 15). This is in good agreement with our previous results. In 6 rats notable hyperplasia of the thyroid epithelium was observed. These pathological alveoli (figs. 17 and 18) consist of solid nests of cells of uneven size and shape, with granular cytoplasm staining bluish with hæmatoxylin and tending to form a syncytium. The nuclei are vesicular and usually larger than those of normal thyroid cells. These structures were observed after treatment with various hormones. In our other experiments (Korenchevsky, Paris and Benjamin) on considerably older (senescent) rats treated simultaneously with sex and thyroid hormones, these structures developed into frank adenomata affecting a part or the whole of the gland (figs. 19, 20 and 21), with cellular anaplasia (figs. 20 and 21).

### *Changes in the parathyroids*

In the control and most of the treated animals the histological structure of the parathyroid glands was normal. In 7 cases, however, the fibrous tissue of the glands was slightly increased; namely in one rat treated with progesterone and thyroid hormone, in one treated with androsterone and desoxycorticosterone, in one receiving A.H. extract, progesterone and thyroid hormone simultaneously and in rats receiving A.H. extract, androsterone and desoxycorticosterone simultaneously. Our experiments on older rats (Korenchevsky, Paris and Benjamin) suggest that an increased development of fibrous tissue is one of the features of ageing of the parathyroids.

## Plate XIV

Hæmatoxylin and eosin. ×160.

Fig. 13.—Control rat. Cubical or slightly higher epithelium ; mostly medium amount of colloid in the follicles. Thyroid 16 mg.

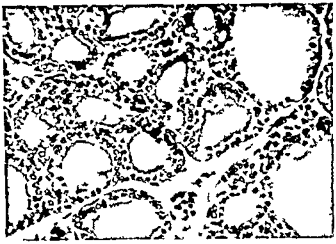
Fig. 14.—Rat treated with thyroid hormone and progesterone. Flattened epithelium and increased amount of colloid, typical of depressed thyroid. Thyroid 17 mg.

Fig. 15.—Rat treated with androsterone and desoxycorticosterone acetate. Very active thyroid, with high epithelium and very little colloid in follicles. Thyroid 20 mg.

Fig. 16.—Rat treated with A.H. extract, thyroid hormone and progesterone. Epithelium similar to that of control rats, but amount of colloid much reduced, Thyroid considerably more active than in fig. 14. Thyroid 18 mg.

ig. 17 and 18.—Small hyperplastic structures, with formation of solid alveoli, of epithelium in the thyroid of rats treated with A.H. extract, progesterone and thyroid hormone (fig. 17), and with androsterone and desoxycorticosterone (fig. 18). Thyroids 21 and 20 mg. respectively.

THYROID



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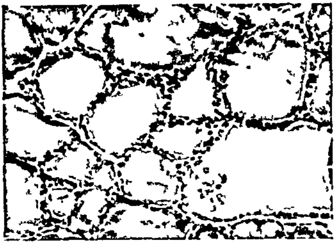

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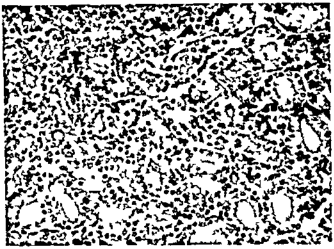

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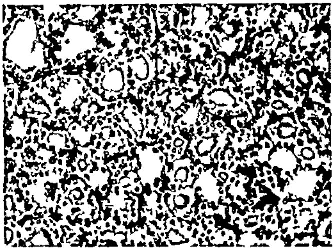

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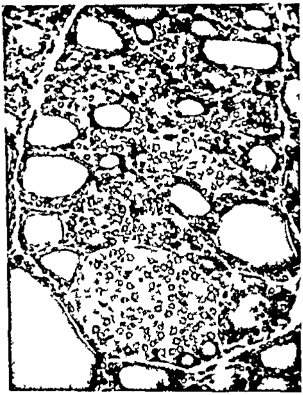

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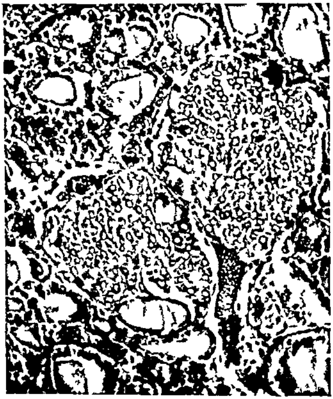

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In contrast to this pathological change, in one rat receiving progesterone and thyroid hormone, the parathyroids were especially active as judged by the larger size of the cells and the presence of mitoses Mitoses, however, were not observed in the other rats of the same group, nor in animals of other groups

*Changes in the thymus*

The literature on the subject and our own results on the effect of androgens in increasing involution of the thymus have already been given (Ross and Korenchevsky, 1941) Similar effects of progesterone in combination with œstradiol have also been recorded (Korenchevsky and Jones, 1948) The effects of desoxycorticosterone and A H hormones were insufficiently studied and have so far given contradictory results No definite or constant effect was obtained with thyroid hormone (Korenchevsky, Hall and Clapham, 1943, Korenchevsky and Jones, 1946, 1948)

In the present experiments, A H extract alone, in both doses, did not produce any effect on the weight of the thymus, but in combination with thyroid hormone (table I, cols 4, 5 and 11), a statistically significant ($P<0\ 02\ 0\ 001$) delay in the involution of the thymus was observed Histologically this was confirmed by the larger size of the thymic lobules and the smaller amount of fibrous and adipose tissue between them With the other hormonal combinations used no significant changes were found in the weight of the thymus Histologically, in rats treated with androsterone and desoxycorticosterone simultaneously, greater atrophy of the thymus was observed than in the control rats, namely smaller lobules, with a greater amount of fat and fibrous tissues Few and very small nests of hyperplastic thymic epithelial cells were observed in some of the rats treated with the combinations which included thyroid hormone or desoxycorticosterone These hyperplastic structures, though of more pronounced degree, have previously been described by us in detail (Ross and Korenchevsky) and confirmed by Plagge (1944)

*Changes in the liver*

Hypertrophy of the liver was obtained in rats after the administration of A H extract by Best and Campbell (1936), Lee and Freeman (1940), Cameron and Carmichael (1941) and Selye and Clarke (1943) Lee and Freeman counted liver cells and found that their hyperplasia was responsible for this enlargement of the liver Best and Campbell found that A H extract increased liver fat considerably, while decreasing general body fat Therefore the enlargement of the liver might be due partly to the fat increase in the organ

Riddle and his co workers (Bates *et al*, 1937) produced hepatic hypertrophy in pigeons by lactogenic A H hormone Li *et al* (1949) did not find this in rats

Progesterone in combination with small and ineffective doses of thyroid hormone or œstradiol, and androgens, also produced liver hypertrophy in ovariectomised rats, less constantly in normal rats (Korenchevsky and co workers 1935, 1937*a* and *b*, 1939, 1946, 1947, 1948 1950)

Thyroid hormone is also an effective hepatotropic factor in both normal and ovariectomised rats (Korenchevsky and co workers, 1943, 1944, 1946)

The effect of desoxycorticosterone was studied by Carnes *et al.* (1941) and Forster *et al.* (1946), who could not find any changes in the liver even by using large doses of this hormone.

In the present experiments, statistically significant hypertrophy of the liver ($P<0{\cdot}02$) was obtained after administration of A.H. extract in large but not in small doses (table I, col. 11). When, however, the small dose was given simultaneously with thyroid hormone, with or without *progesterone*, the hypertrophy became significant ($P<0{\cdot}01$; cols. 4 and 5). With this combination the larger doses produced the greatest increase in weight of the liver ($P<0{\cdot}001$; col. 11). A.H. extract in combination with desoxycorticosterone or androsterone, or the latter hormones alone, produced no significant enlargement of the liver.

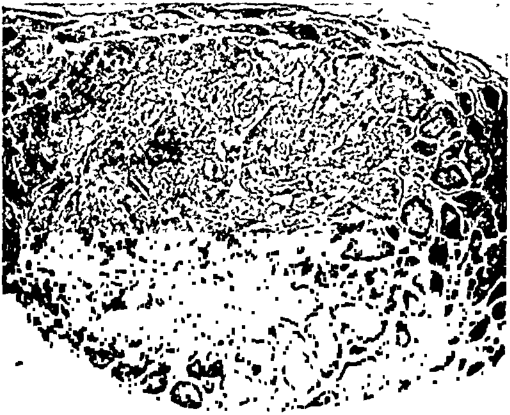
A synergistic relationship between A.H. and thyroid hormones is indicated by the absence of significant hepatic hypertrophy with a combination of progesterone and thyroid hormone (table I, col. 2), while a significant hypertrophy was obtained after the addition to this combination of a small dose of A.H. extract (col. 5). Previously it had been found that such small doses of thyroid hormone as were used in the present experiment do not produce definite liver hypertrophy (Korenchevsky and Jones, 1946, 1948), but the larger doses do (Korenchevsky, Hall and Clapham, 1943). Thus small and comparatively inactive doses of the hormones of the anterior lobe and thyroid exert a definite synergistic action when applied together.

Comparison of photomicrographs of the liver cells in various groups demonstrates definite enlargement in two groups only:—in rats injected simultaneously with a small dose of A.H. extract and thyroid hormone and in rats injected with a large dose of A.H. extract. Therefore the hypertrophy of the liver observed in our experiments should be due chiefly to hyperplasia and not to hypertrophy of the cells.

Two kinds of pathological change were observed. (1) Small and infrequent foci of round-cell infiltration were recorded, usually around small blood vessels, in approximately equal numbers in both control and treated groups. (2) Focal hyperplasia of the smallest bile ducts was observed in 9 rats treated with various hormones (table III, p. 125). These hyperplastic foci were minute in size, consisting of clumps of 4-7 tubules. Only one or two foci were found in whole sections of small lobes of the liver. These small foci and some of larger size (fig. 22) were occasionally or even frequently found in normal untreated rats at the more advanced age of 20-28 months (Korenchevsky, Paris and Benjamin). Most probably they are a feature of old age.

Histologically similar structures have been observed in the human liver and, when developed, have been described as multiple adenomata of the bile ducts (Ewing, 1940, p. 750). Transitions to cystadenoma or adenocarcinoma may occur.

THYROID ADENOMA AND HYPERPLASIA OF HEPATIC BILE DUCTS



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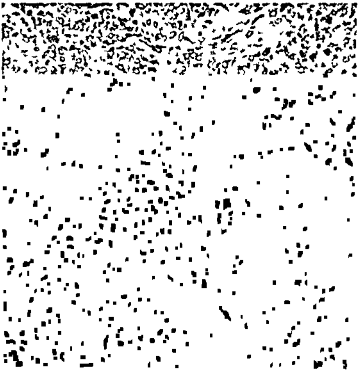

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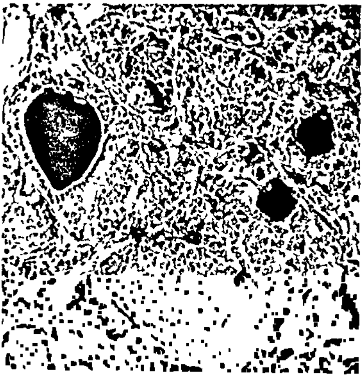

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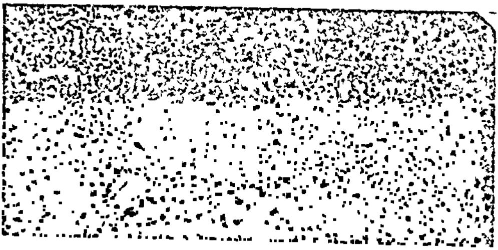

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## PLATE XVI

Hæmatoxylin and eosin

FIG. 23.—Untreated control rat. Normal structure of exocrine acinar tissue. ×185.

FIG. 24.—Rat treated with A.H. extract 3 mg. and thyroid hormone. ×185.

FIG. 25.—Rat treated with A.H. extract 6 mg., progesterone and thyroid hormone. ×185.

FIG. 26.—Rat treated with A.H. extract, androsterone and desoxycosticosterone acetate. ×185.

In figs. 24-26 the number of larger acini with larger cells is increased as compared with that in the control rat (fig. 23).

FIG. 27.—Rat treated with A.H. extract 3 mg. and desoxycorticosterone acetate. At the top, right-hand side, and in the right-hand bottom corner, normal exocrine acinar tissue. In the left-hand bottom corner, part of a normal islet of Langerhans. Between these normal tissues, a triangular area shows transition from normal exocrine acini into widely luminated acini lined by smaller flattened cells, evenly and faintly stained, with smaller and usually flattened nuclei containing less chromatin than the normal cells. ×185.

FIG. 28.—Rat treated with A.H. extract, androsterone and desoxycorticosterone acetate. Three lobules present an adenoma-like transformation, with comparatively few acini still in the transitional stage. There is slight round-cell infiltration. In the right-hand bottom corner there is a small portion of normal pancreatic tissue. ×65.

## PLATE XV

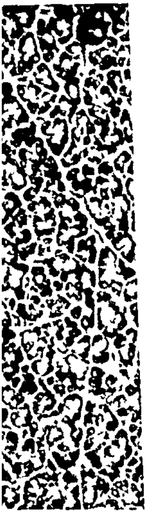
Hæmatoxylin and eosin

FIG. 19.—Female rat, aged about 27 months, treated with testosterone propionate. In rats the thyroid consists of two large lobes, right and left, joined by a very narrow thin isthmus. An adenoma has transformed nearly the whole of the right lobe, leaving a few normal follicles on the right-hand side and top edges, recognisable by their normal colloid content. ×40.

FIG. 20 and 21.—Another female rat, about 27 months old, treated with progesterone, œstradiol B.B. and thyroid hormone. An adenoma has spread through the whole lobe. The photographs (×140), taken from two different parts of the tumour, show that the follicular arrangement, although distorted, is partly retained, partly broken (fig. 20), with the cells spreading irregularly in all directions. Colloid remains in four places in fig. 21. Cells and nuclei are of various sizes and shapes. In places the structure resembles that of a spindle-cell sarcoma. Thyroid 26 mg.

FIG. 22.—Liver of control intact male rat aged about 21 months. Amongst the normal hepatic parenchyma are two portal tracts showing well-marked bile-duct hyperplasia. ×110.

# PANCREAS



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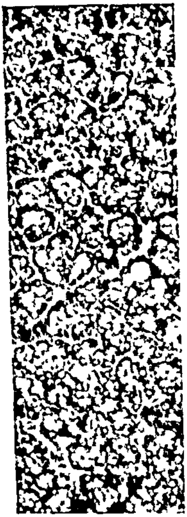

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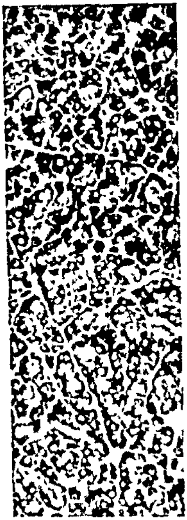

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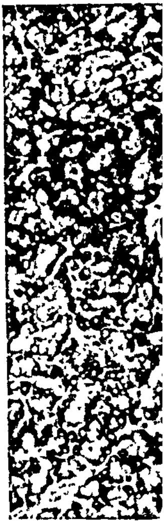

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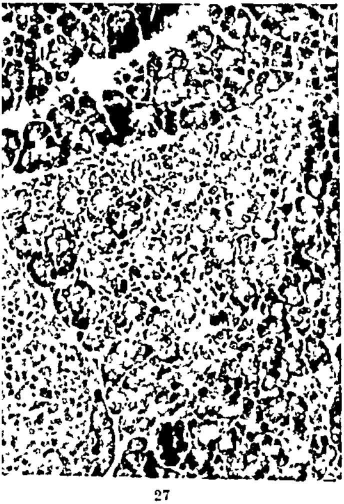

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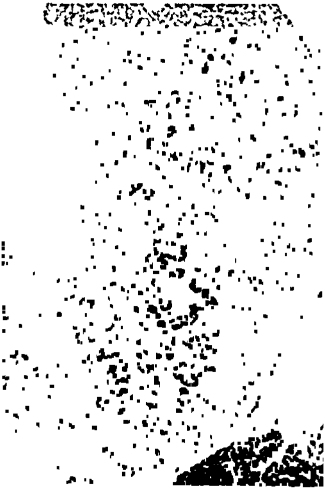

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### *Changes in the kidneys*

Wilkins *et al.* (1935) and Cameron and Carmichael (1941) produced renal hypertrophy by the injection of A.H. extracts. Selye and co-workers (Selye and Clarke, 1943 ; Selye and Pentz, 1943 ; Hall *et al.*, 1946 ; Dontigny *et al.*, 1948) also produced hypertrophy of the kidneys, but at the same time severe nephrosclerotic lesions, when very large doses of A.H. extracts or desoxycorticosterone acetate were injected into rats. Reiss (1944) found degenerative and necrotic lesions within 12 hours of the injection of a large dose of the corticotropic A.H. extract.

Contrary to the observations of Selye and co-workers, Forster *et al.* (1940), after the daily injection of rats with 2·5 mg. of desoxycorticosterone for 87 days, *i.e.* large doses, found only slight degenerative changes in some tubules and an increase in hyaline and granular casts. Carnes *et al.* (1941), in a similar experiment, found no lesion in the kidneys.

Korenchevsky and co-workers, in the papers mentioned in the section on the liver, summarised the literature of the subject and their own experiments, in which they observed considerable hypertrophy of the kidneys after the administration of androgenic and thyroid hormones and progesterone. While thyroid hormone produced this effect in both normal and ovariectomised rats, androgenic hormones, and in particular androsterone, had much less effect and sometimes no effect at all in normal intact rats. Measurement of the convoluted tubules and mitotic counts demonstrated that in the treated rats the renal enlargement was a true hypertrophy, being produced chiefly by overgrowth of individual renal cells.

In the present experiments, significant hypertrophy of the kidneys was not obtained with A.H. extract alone (table I, cols. 3 and 10) nor with a combination of progesterone and thyroid hormone (col. 2). When, however, these three hormones were administered together (cols. 5 and 11), or only A.H. extract and thyroid hormone (col. 4), a statistically significant hypertrophy ($P<0{\cdot}02$-$0{\cdot}01$) was observed. These data indicate a synergistic action of A.H. hormones with thyroid hormone on the kidneys.

The combination of A.H. extract and androsterone (col. 7) did not produce any effect on the weight of the kidneys, but with the addition of desoxycorticosterone (col. 8) there was a significant hypertrophy ($P<0{\cdot}02$). In this case a synergistic relationship was in operation between androsterone and desoxycorticosterone as the data in col. 9 indicate ($P<0{\cdot}05$) ; especially, since a significant change was not obtained with the combination of A.H. extract and desoxycorticosterone (col. 6).

Histological measurement of proximal convoluted renal tubules and their lumina by the method of paper replicas has shown that hypertrophy of the tubules was obtained, not only in the case of rats with significantly increased kidney weights (table II, lines 4, 5, 8 and 10), but also in those in which the increase in weight was not significant (line 9 ; $P>0{\cdot}05$).

It is noteworthy that not only did the tubular cells become hypertrophied, but the tubular lumina also were significantly enlarged ($P<0{\cdot}05$-$0{\cdot}01$ ; the groups in table II, lines 6, 9 and 10). These measurements therefore indicate that the enlargement of the kidneys in the respective groups was the result of a true renal hypertrophy.

TABLE II

*Effect of hormones on renal proximal convoluted tubules and their lumina*

| | Groups | Average weight (mg.) of paper replicas of tubules | |
|---|---|---|---|
| | | Tubule without lumen | Lumen |
| 1 | Controls . . . . . . . . | 5·46 | 0·92 |
| 2 | Progesterone+thyroid hormone . . . | 5·87 | 1·44 |
| 3 | A.H. extract alone, 3 mg. . . . . | 5·73 | 1·39 |
| 4 | A.H. extract+thyroid hormone . . . | 6·65 | 1·32 |
| 5 | A.H. extract+thyroid hormone+progesterone | 6·69 | 1·69 |
| 6 | A.H. extract+androsterone . . . | 6·26 | 1·41 |
| 7 | A.H. extract+androsterone+desoxycorticosterone | 5·84 | 1·50 |
| 8 | Androsterone+desoxycorticosterone . . | 6·41 | 1·61 |
| 9 | A.H. extract alone, 6 mg. . . . . | 6·59 | 1·83 |
| 10 | A.H. extract, 6 mg.+thyroid hormone+progesterone | 6·49 | 1·82 |

Pathological changes in the form of round-cell infiltration and œdema of the glomeruli were observed in both control and treated rats. In the controls, however, the foci of cellular infiltration were not only very small but also rare, only one or two of them being seen in a whole cross section of the kidney. Moreover they were found only in 3 out of 8 control rats. Œdema of glomeruli was observed in 1 control rat only, and one or two œdematous glomeruli in the transverse section of the whole kidney.

In the groups treated with combinations in which desoxycorticosterone was present, or in which A.H. extract, especially in a large dose, was administered simultaneously with progesterone and thyroid hormone, the foci of cellular infiltration were definitely increased in number but only rarely in size. For example, foci of infiltration were found in the kidneys of every rat in the group " A.H. extract+desoxycorticosterone ", and the group " large dose of A.H. extract+progesterone+thyroid hormone ". In addition, in 2 out of 7 rats in the latter group, tiny spots of degeneration, and in 1 rat tiny spots of necrosis were found in the cortical part of the kidneys, there being from one to three spots in the whole cross section of the kidney.

Out of all the rats treated, only one—an animal belonging to the group treated simultaneously with A.H. hormone, androsterone and desoxycorticosterone—showed foci of cellular infiltration. Although very small, these were spread around so many tubules that the lesion could be regarded as the beginning of a mild interstitial nephritis.

### *Changes in the spleen*

Hoskins (1916), Herring (1917), Cameron and Carmichael (1920-21), Korenchevsky, Hall and Clapham (1943) and Korenchevsky and Jones (1946, 1948) obtained hypertrophy of the spleen after administration of thyroid hormone in sufficiently large doses. The effect of androsterone was uncertain, producing mostly an increase in weight of the spleen in ovariectomised but not in normal rats (Korenchevsky, Hall and Burbank, 1939; Korenchevsky and Jones, 1946; Korenchevsky, Paris and Benjamin, 1950). Progesterone (Selye, 1940*b*; Korenchevsky and Jones, 1948) and desoxycorticosterone acetate (Selye, 1940*b*) apparently do not produce definite splenic changes.

In the present experiments, a statistically significant increase in weight of the spleen ($P<0{\cdot}01$-$0{\cdot}001$) was obtained only in the groups simultaneously injected with A.H. extract and thyroid hormone, with or without progesterone (table I, cols. 4, 5 and 11). Since progesterone with thyroid hormone alone (col. 2) did not produce this effect, a synergistic action of anterior lobe hormones with thyroid hormone is indicated.

Histologically the structure of these hypertrophied spleens was normal except for the apparently increased size of the Malpighian bodies.

### *Changes in the heart*

The effect of thyroid hormone on the heart has been investigated and the literature surveyed in previous papers (Korenchevsky, Hall and Clapham, 1943; Korenchevsky and Jones, 1946; Korenchevsky, 1950; Korenchevsky, Paris and Benjamin, 1950). Thyroid hormone produces hypertrophy of the heart, and, with larger but apparently well-tolerated doses, various degrees of myo-, endo- or pericarditis in some of the animals. Severe lesions were obtained with toxic doses of this hormone.

Androsterone (Korenchevsky, Hall and Burbank, 1939; Korenchevsky and Jones, 1946) is also able to produce hypertrophy of the heart; progesterone (Selye, 1940*b*; Korenchevsky and Jones, 1948) has apparently no definite action. Desoxycorticosterone acetate and A.H. extracts in very large doses (Selye and Pentz, 1943; Hall *et al.*, 1946) produce hypertrophy, with myocardial lesions in the form of infiltration with Aschoff cells, lymphocytes, fibroblasts and leucocytes, and very small foci of necrosis or sclerotic scars. Carnes *et al.* (1941) and Forster *et al.* (1946), also using large doses of desoxycorticosterone acetate, could not find any pathological changes in the heart of rats or (Foster *et al.*) dogs.

In the present experiments statistically significant hypertrophy was obtained in all animals receiving thyroid hormone, but was more pronounced in combinations with A.H. extract (table I, cols. 4, 5 and 11; $P<0{\cdot}01$-$0{\cdot}001$) than without it (col. 2; $P<0{\cdot}03$). Similarly, while desoxycorticosterone and androsterone alone (col. 9) or andro sterone+small dose of A.H. extract (col. 7) produced a non-significant increase in the weight of the heart, the increase was significant when all three hormones were injected simultaneously (col. 8; $P<0{\cdot}01$). This indicates a synergistic action on the heart between anterior lobe hormones and thyroid hormone, and between anterior lobe hormone, androsterone and desoxycorticosterone.

measurements therefore indicate that the enlargement of the kidneys in the respective groups was the result of a true renal hypertrophy.

TABLE II

*Effect of hormones on renal proximal convoluted tubules and their lumina*

| | Groups | Average weight (mg.) of paper replicas of tubules | |
|---|---|---|---|
| | | Tubule without lumen | Lumen |
| 1 | Controls . . . . . . . . | 5·46 | 0·92 |
| 2 | Progesterone+thyroid hormone . . . | 5·87 | 1·44 |
| 3 | A.H. extract alone, 3 mg. . . . . | 5·73 | 1·39 |
| 4 | A.H. extract+thyroid hormone . . . | 6·65 | 1·32 |
| 5 | A.H. extract+thyroid hormone+progesterone | 6·69 | 1·69 |
| 6 | A.H. extract+androsterone . . . | 6·26 | 1·41 |
| 7 | A.H. extract+androsterone+desoxycorticosterone | 5·84 | 1·50 |
| 8 | Androsterone+desoxycorticosterone . . | 6·41 | 1·61 |
| 9 | A.H. extract alone, 6 mg. . . . . | 6·59 | 1·83 |
| 10 | A.H. extract, 6 mg.+thyroid hormone+progesterone | 6·49 | 1·82 |

Pathological changes in the form of round-cell infiltration and œdema of the glomeruli were observed in both control and treated rats. In the controls, however, the foci of cellular infiltration were not only very small but also rare, only one or two of them being seen in a whole cross section of the kidney. Moreover they were found only in 3 out of 8 control rats. Œdema of glomeruli was observed in 1 control rat only, and one or two œdematous glomeruli in the transverse section of the whole kidney.

In the groups treated with combinations in which desoxycorticosterone was present, or in which A.H. extract, especially in a large dose, was administered simultaneously with progesterone and thyroid hormone, the foci of cellular infiltration were definitely increased in number but only rarely in size. For example, foci of infiltration were found in the kidneys of every rat in the group " A.H. extract+desoxycorticosterone ", and the group " large dose of A.H. extract+progesterone+thyroid hormone ". In addition, in 2 out of 7 rats in the latter group, tiny spots of degeneration, and in 1 rat tiny spots of necrosis were found in the cortical part of the kidneys, there being from one to three spots in the whole cross section of the kidney.

Out of all the rats treated, only one—an animal belonging to the group treated simultaneously with A.H. hormone, androsterone and desoxycorticosterone—showed foci of cellular infiltration. Although very small, these were spread around so many tubules that the lesion could be regarded as the beginning of a mild interstitial nephritis.

In other experiments (Korenchevsky, Paris and Benjamin, 1950) on senescent rats aged 22-27 months injected with sex and thyroid hormones, these structures were more frequent and better developed.

TABLE III

*Number of rats in the various groups showing hyperplastic changes in various organs*

| Groups of rats | | No. of rats in group | Number of structures in | | | | |
|---|---|---|---|---|---|---|---|
| | | | thyroid | adrenals | pancreas | thymus | liver |
| 1 | Controls . . . | 8 | 0 | 0 | 0 | 0 | 0 |
| 2 | Progesterone+thyroid hormone | 7 | 0 | 0 | 1 | 2 | 1 |
| 3 | A.H. extract, 3 mg. . | 7 | 0 | 0 | 1 | 0 | 1 |
| 4 | A.H. extract, 3 mg.+ thyroid hormone | 7 | 0 | 0 | 1 | 1 | 0 |
| 5 | A.H. extract, 3 mg.+ thyroid hormone+progesterone | 7 | 0 | 0 | 0 | 2 | 3 |
| 6 | A.H. extract, 3 mg.+ desoxycorticosterone | 7 | 1 | 0 | 1 | 0 | 0 |
| 7 | A.H. extract, 3 mg.+ androsterone | 8 | 0 | 1 | 1 | 0 | 0 |
| 8 | A.H. extract, 3 mg.+ androsterone+desoxycorticosterone | 9 | 3 | 0 | 3 | 1 | 2 |
| 9 | Androsterone+desoxycorticosterone | 7 | 2 | 0 | 0 | 2 | 0 |
| 10 | A.H. extract, 6 mg. . | 6 | 0 | 0 | 0 | 0 | 2 |
| 11 | A.H. extract, 6 mg.+ thyroid hormone+progesterone | 7 | 0 | 0 | 1 | 2 | 0 |
| | Total number . . | 80 | 6 | 1 | 9 | 10 | 9 |

To a lesser degree and less frequently they have been observed even in control senescent rats. It appears therefore that this pathological tendency to the development of adenoma-like structures has a connection with and is perhaps one of the features of senescence in rats.

Adenomas and cystadenomas have been observed in the human pancreas but are comparatively rare (Ewing, p. 762). They usually arise from the ducts, but an origin from the exocrine acinar cells, or even from the cells of Langerhans islets has also been recorded.

### *Changes in the hypophysis*

No histological investigation of the hypophysis has been made in the present experiments. A statistically significant decrease ($P<0{\cdot}02$-$0{\cdot}01$) in the weight of the hypophysis was found in rats treated with a small dose of A.H. extract plus thyroid hormone (*table I*, col. 4) or androsterone (col. 7). A combination of androsterone and desoxycorticosterone also produced a significant decrease of the weight (col. 9; $P<0{\cdot}02$).

As regards anterior lobe hormones alone, in the present experiment the small dose used had no effect (col. 3), while the large dose had a definite hypertrophying action (col. 10; $P<0{\cdot}02$).

Histologically, none of the severe pathological changes which followed the administration of very large doses of A.H. extracts and desoxycorticosterone acetate (Selye and co-workers) and toxic doses of thyroid hormone was obtained in the present experiments; apparently the doses used were non-toxic for the heart. The diet on which the rats were fed may have been partly responsible for this. Only in two rats of one group, simultaneously injected with a small dose of A.H. extract, androsterone and desoxycorticosterone acetate, were tiny foci of round-cell infiltration found in the myocardium—one or two in a whole transverse section of the heart.

### *Changes in the pancreas*

Cameron (1926) gave a full review of the previous literature on the effects of thyroid hormone on the pancreas, together with the results of his own careful experiments. He confirmed the hypertrophy of the pancreas observed by previous workers, and found also that thyroid hormone caused an increase in the ratio of Langerhans islet tissue to exocrine tissue. Florentin and Watrin (1931), and Richardson and Young (1937-38) confirmed Cameron's results. The latter authors found that the presence of giant islets did not always indicate proliferative activity of these structures, since in some cases giant islets were unaccompanied by an increase in the islet/acinar ratio. No reliable results were obtained with androsterone, progesterone and desoxycorticosterone.

In the present experiments, assay of the amount of islet tissue was not attempted. In photomicrographs, as compared with control rats (fig. 23), the number of larger exocrine acini appeared to be greater in the rats to which A.H. extract was administered in combination with thyroid hormone (fig. 24), with thyroid hormone and progesterone (fig. 25), or with androsterone and desoxycorticosterone (fig. 26).

Hyperplastic structures were observed in the exocrine pancreatic parenchyma which were not found in control animals, In most of the treated groups only a single example of this change was encountered (table III), while the size of the areas involved was very small in all the pancreases except three. In two of these exceptional cases the greater part of a small pancreatic lobule was affected (fig. 27); in the third, three lobules showed this change (fig. 28), which presented an adenoma-like formation. The change consists in a loss of the normal granulation of the exocrine cells and in the disappearance of any distinction between their outer hæmatoxylinophilic and inner eosinophilic zones. The cells become flatter, with smaller and usually flattened nuclei, the lumina in consequence becoming much increased in size (fig. 27). In some of the affected pancreases these structures were infiltrated with round cells and fibroblasts (fig. 28), in others, infiltration was practically absent (fig. 27).

### *Pathological effects*

Very severe lesions in the kidneys and heart, as obtained by Selye and co-workers, were not observed in our experiments. There were practically no cardiac lesions in our treated rats, while in the kidneys, round-cell infiltration and œdema of glomeruli were increased, but only slightly as compared with the controls. In one of the treated rats the cellular infiltration, although still small in size, had spread between so many tubules as to suggest the early stages of a mild interstitial nephritis.

The difference between our results and those obtained by Selye could be explained by differences in the diet and in the doses used. Selye and his co-workers injected enormous daily doses, for example 20-40 mg. of A.H. extract (Dontigny *et al.*, 1948) and 6 mg. desoxycorticosterone acetate. Corresponding daily doses for human beings would be of the order of 6-12 g. of A.H. extract and 1·8 g. of desoxycorticosterone acetate.

Hormones and several other substances, vitally necessary in physiological and harmless amounts, may become deadly poisons when used in huge doses. Such amounts are unlikely to occur naturally in rats and it is doubtful whether they could even be secreted in cases of pathological hypersecretion in man.

Moreover Selye and co-workers, in order to produce severe nephrosclerotic or cardio-vascular lesions, usually administered to the rats, besides hormones, large amounts of sodium chloride or high protein diets ; or the treated rats were castrated or unilaterally nephrectomised. They themselves (Hay *et al.*, 1948) demonstrated what a very aggravating effect these additional measures have. For instance, by decreasing the protein content in the diet they were able to prevent completely the nephrosclerotic effect of A.H. hormones in rats. On the other hand, nephrosclerotic lesions could be produced by some protein-rich diets alone without the administration of any hormones.

Even for the correct interpretation of these comparatively slight lesions of various organs in the present experiments, it is necessary to emphasis that the doses used, although much smaller than those of Selye and co-workers, were still large doses.

Other pathological changes—hyperplastic structures in the liver, adrenals, thyroid and pancreas were also slight in the present experiments. Their total number, however, was considerable. Various changes of this kind occurred in different organs in 35 out of 72 treated rats (table III). From the results of their own investigations on human hyperpituitarism, especially acromegaly, and from a study of the literature, Cushing and Davidoff (1927, p. 96) came to the conclusion that hyperpituitarism tends to promote adenomatous formations in some glands (thyroid, parathyroid, adrenals and pancreas).

Our present results and those obtained on older rats, however, clearly indicate that hypersecretion, not only on the part of the anterior hypophysis but also of the gonads, thyroid and adrenal cortex, has the same effect. In the present experiment, for example, in groups 2 and 9 (table III), which were not treated with A.H. extract, the occurrence of these epithelial changes was even more frequent than in the remaining groups treated with A.H. extract (about 57 and 47 per cent. respectively).

### *Pathological changes and ageing*

In the present experiments on rats about 17 months old some pathological changes were also present in the controls (infiltrations in the kidneys and liver), but these were fewer and slighter than in the treated rats.

In our other experiments on older rats, aged 20-27 months (Korenchevsky, Paris and Benjamin), the controls all showed pathological changes, which were more frequent and often more severe. It was therefore suggested that these lesions, or the tendency and predisposition to their development, might be a feature of ageing. This was apparently confirmed by the fact that doses of hormones similar to those used in the present experiment usually produced, in older rats, more frequent and more severe changes. For example, hyperplastic formations in the thyroid, adrenals, thymus, liver and pancreas developed in some of these rats into more pronounced adenoma-like structures or even, in the thyroid, into frank adenomata (figs. 19-21). Similar formations were observed by Korenchevsky and Hall (1940) in the uterine mucosa of ovariectomised rats injected with androgens and œstrogens simultaneously. These rats were not more than 214 days old, but according to our previous investigations ovariectomy hastens the process of ageing in rats.

It is noteworthy that minute single or multiple nodular hyperplasias or adenoma-like structures are also associated with ageing in man (Rolleston, 1936, p. 373; Ewing, 1940, pp. 830-831). In the adrenals of adult or old persons, for example, they are discovered at autopsy as incidental findings in about 35 per cent. of cases, and Ewing emphasises that all gradations exist between simple local adrenocortical hyperplasia and true adenoma formation.

### *Stimulating effects of hormones and ageing*

In contrast to the induction of pathological lesions by hormones, their stimulating and hypertrophying effects must be emphasised. These changes occur in the vital organs of rats, especially after combined hormonal treatment. In this limited sense, the stimulating and hypertrophying effects of hormones appear as anti-ageing actions, since, with ageing, all organs ordinarily undergo relative hypoplasia (Korenchevsky, 1942) and finally atrophic and degenerative changes.

This fact, however, does not necessarily mean that there is a truly beneficial effect on the process of ageing as a whole. It might, on the contrary, indicate the development of splanchnomegalic monstrosity. Many morphological, physiological and biochemical experiments are necessary in order to define the true significance of any apparently ageing or anti-ageing factors. This problem is considered in detail elsewhere (Korenchevsky, Paris and Benjamin, 1950).

## SUMMARY

1. An experiment was performed on 80 ageing normal rats in order to investigate the effect of hormones of the anterior hypophysis (A.H.) administered alone or in various combinations with androsterone, progesterone, desoxycorticosterone acetate and thyroid hormone.

2. In the A.H. extract used as a source of hormones of the anterior hypophysis there were present gonadotropic hormones for the ovaries, and somatotropic hormones for the preputial glands, thyroid, adrenals, thymus, liver, kidneys, spleen and heart.

3. The presence of these hormones in the A.H. extract was ascertained by their hypertrophying or stimulating, or their synergistic or co-operative effects on the respective organs.

4. A synergistic or co-operative relationship between A.H. hormones and thyroid hormone was observed in their action on the ovaries, preputial glands, adrenals, thymus, liver, kidneys, spleen and heart.

5. A.H. hormones were able to neutralise more or less completely the depressing effect of thyroid hormone on the thyroid gland, but not the atrophying effect of androgens on the adrenals.

6. Synergistic or antagonistic relationships between some of the other hormones used were also recorded.

7. Pathological lesions in some of the treated rats took the form of focal infiltration, chiefly lymphocytic, of the kidneys, liver and pancreas, of the formation of epithelial hyperplastic structures in the thyroid, thymus, liver and pancreas, or of hypoplasia of the ovaries and adrenals. In nearly all cases the first two groups of lesions were slight and, in addition, some of them were present also in the control rats, though less frequently.

8. Some apparently anti-ageing (stimulating and hypertrophying) properties of the hormones used are discussed; and the role of senescence in producing and intensifying the pathological lesions observed is considered.

9. It is emphasised that many morphological, physiological and biochemical investigations are necessary in order to ascertain the true nature of any apparently ageing or anti-ageing factors.

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# GROSS TUMOUR EMBOLISM OF THE AXILLARY ARTERY BY MALIGNANT SYNOVIOMA

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(Plates XVII-XIX)

That tumour emboli of macroscopic dimensions may be carried in the systemic veins to be arrested in the right chambers of the heart, the pulmonary arteries or lungs, and in the portal vein to the liver is a well known but rare occurrence. Emboli of this kind are still more rarely encountered in the arterial system (Willis, 1934). They may result from neoplastic invasion of pulmonary veins or the left chambers of the heart, or from paradoxical (crossed) embolism. In the few recorded cases, the sites of arrest have been different and, with one exception, the finding was *post mortem*.

The only case in the literature of gross arterial tumour embolism recognised during life is that of Groth (1940). His patient, a woman of 36 who two years previously had had her right leg amputated for sarcoma of the tibia, presented with obstruction of the left common femoral artery by a tumour embolus. This was removed successfully and she survived for one month after the embolectomy. At necropsy, pulmonary metastases were found to have invaded main pulmonary veins.

Kaufmann (1929) has referred briefly to the finding at necropsy, in a woman of 25, of gross invasion of pulmonary veins by metastases from chorionic carcinoma and of macroscopic tumour emboli in arteries of the spleen and kidneys producing infarcts in these organs. Thompson and Evans (1929-30) described a case of paradoxical tumour embolism through a patent foramen ovale in a man of 25 with bilateral malignant testicular teratomas. There were no pulmonary metastases but growth from a mass of tumour attached to the right surface of the atrial septum projected through the foramen ovale into the left atrium. Free and attached tumour masses were present in the left ventricle and gross tumour emboli were found in coronary and middle cerebral arteries.

It is doubtful if the case described by Till and Fairburn (1947-48) is acceptable as one of massive neoplastic embolism. Their patient, a man of 50 with bronchial carcinoma, developed occlusion of the right femoral artery followed by gangrene of the foot. At necropsy, the obstructing mass consisted of thrombus which on microscopic examination was found to include tumour tissue only in a restricted part, with an attachment to the wall immediately proximal to the origin of the profunda femoris artery. No gross invasion of vessels was found in the thoracic viscera. It is therefore possible that the femoral occlusion may have developed from microscopic rather than gross tumour embolism, and the finding by Branwood and Glazebrook (1946) of a tumour nodule implanted on an atheromatous patch in the aorta in a case of mixed-cell sarcoma of the diaphragm is of interest in this connection.

In a child of 10 years, Young and Hunter (1947) found soft, mucinous, polypoid masses attached to the wall of the left ventricle and an embolus of similar tissue occluding the abdominal aorta from the cœliac to the renal arteries. Though the authors conclude that this was a cardiac myxoma rather than organising thrombus, it is questionable whether the case can be accepted as an instance of arterial tumour embolism because of the equivocally neoplastic nature of such tumours.

## CASE REPORT

In our patient there was gross tumour embolism of the left axillary and brachial arteries, confirmed by histological examination of the embolectomy specimen, and, at necropsy, massive permeation of pulmonary veins.

### *Clinical history*

S. A. O., a woman of 64 years, was admitted to the Royal Cancer Hospital as an emergency case on 15th December 1948, with a 9-hours' history of severe pain in the left arm. She had noted that the limb had changed colour, becoming first white and then blue. She had been receiving a course of X-ray therapy as an out-patient following the removal, at another hospital, of a secondary malignant synovioma from the region of the left scapula. A radiograph on 24th November 1948, had shown an opacity of doubtful significance in the right lung.

*Past history.* In October 1945 a tumour of the left knee which had been present for 8 months was excised. It appeared to be entirely extra-capsular and was described as a softish, homogeneous, solid tumour of tangerine size, sharply circumscribed from the surrounding fatty tissue and yellowish on cut surfaces. The histological diagnosis was synovioma (Dr E. M. Martland). During September 1946, X-ray therapy was given for a recurrence of the knee tumour and in September 1948, a further recurrence in the same site was excised. Five days later a tumour was noticed in the left scapula. This was treated by excision followed by X-ray therapy.

*On examination,* there were no physical signs of cardiac abnormality. The blood pressure was 140/90. In the region of the inferior angle of the left scapula there was a healed scar. The left arm was cyanosed, with white areas of mottling, and was cold below the middle of the upper arm. Pulsation was absent in the radial, brachial and axillary arteries but present in the subclavian. A further radiograph of the chest showed the same opacity in the right lung and also a more peripheral shadow.

*Operation* (*T.G.E.L.*) *15th December* 1948. As there was no obvious source of the embolus, the axillary artery was explored, under general anæsthesia, through the lower limb of a Fiolle-Delmas incision. The artery was opened at the origin of the subscapular branch, revealing a mass of friable tissue, which was removed. A good efflux of blood was obtained from both ends but there was no return of the radial pulse. Because of the tumour-like appearance of the embolus, further exploration was considered inadvisable. Heparin therapy was begun.

*Pathological report on embolus.* This bilobed, soft white and dark red fragment (larger lobe 0·6 cm. diam.) was found to consist of œdematous spindle-cell growth of unspecifiable nature, accompanied by blood-clot.

*Progress.* Gradual deterioration ensued and death occurred on 16th December 1948. The condition of the arm appeared unchanged.

## GROSS TUMOUR EMBOLISM OF AXILLARY ARTERY

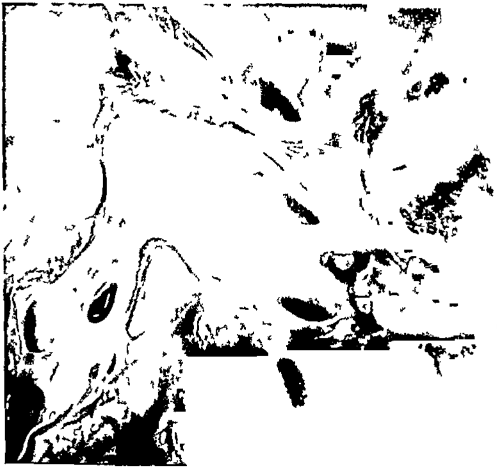

FIG 1.—Distal extremity of growth permeating pulmonary vein in left lung Natural size

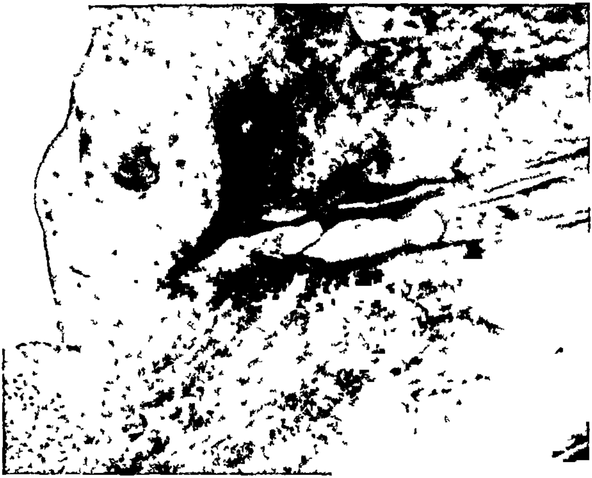

FIG 2.—Right pulmonary metastasis showing permeation of pulmonary vein × 2.5

## *Necropsy findings*

A well-built, fairly obese woman with scars over the left knee and left scapula. There was diffuse dark blue discolouration of the skin of the left arm, which however was paler and more patchy in the neighbourhood of the shoulder: similar patchy discolouration was present over the left side of the chest. A recent transverse infraclavicular (Fiolle-Delmas) incision had been closed by clips.

No residual growth was present at the knee—the site of the primary tumour—nor about the site of the treated scapular metastasis.

Only two pulmonary metastases were present, one near the apex of each upper lobe. They were rounded, and the cut surfaces were firm, white and greyish-white and slightly mucinous. From the tumour (2·2 cm. diameter) in the left lung, a firm cord of growth 5·0 cm. in length extended along the lumen of a pulmonary vein, gradually increasing in diameter to 1·0 cm., when it ended abruptly with an irregular, broken-off free extremity (fig. 1). It consisted of firm white and translucent grey tissue which was smooth-surfaced where it was not attached to the vessel wall. Lying free in a large pulmonary vein at the hilum of the left lung was a twisted clot 5·0 cm. long and from 0·3 to 0·8 cm. wide. The tumour in the upper lobe of the right lung was similar, though smaller (1·6 cm. diameter), and its finer intravascular extension, 2·1 cm. long and up to 0·3 cm. diameter had a smooth, bulbous distal extremity (fig. 2).

There was no tumour within the cavities of the heart but in the posterior wall of the left ventricle was a single, ill-defined metastasis (0·4 cm. diameter) which did not reach either endocardium or epicardium. A small valvular patent foramen ovale allowed the passage of a thick probe. The left axillary artery (fig. 3) was filled with clot from just proximal to the origin of the subscapular artery onwards into the brachial artery for a total distance of 11·0 cm. In its distal 1·5 cm. the "clot" consisted largely of white tumour tissue but the rest was dark red in colour.

On the surface of the parietal region of the left cerebral hemisphere there was a bulging dark red and greyish-brown tumour 2·2 cm. in main diameter, and there were soft, ill-defined, hæmorrhagic areas superficially in the right parietal region and in the left cerebellar hemisphere. The thyroid contained one nodule of secondary growth 1·2 cm. in diameter. The only other metastases were single soft white deposits, 0·8 and 0·6 cm. diameter, in the second and third lumbar vertebral bodies. The thoracic duct and all lymph nodes were normal. Apart from purulent bronchitis and bronchopneumonia there were no other significant changes in the body.

## *Histology*

*Tumour excised in* 1945. Sections of the periphery of the primary tumour, where it extended into the subcutis, were available for examination. Though the margin appeared well defined to the naked eye, the sections show infiltrative growth at the periphery with extension through a condensation of surrounding fibrous tissue into subcutaneous fat. The growth consists of cellular tissue through which many microscopic clefts are irregularly scattered (fig. 4). Hæmorrhagic, œdematous and necrotic areas are small and infrequent. Within the tumour there is very little collagenous tissue. There is considerable pleomorphism but most of the tumour cells are spindle-shaped, having abundant cytoplasm of indefinite outline and plump, ovoid or elongated, pale-staining nuclei, each with a distinct nucleolus. The cells are arranged partly in vague drifts and partly in small compact clumps with intervening clefts (fig. 5). In looser areas, cytoplasmic outlines are clearer and the cells are fusiform, stellate or polygonal. Fine vacuolation and foaminess of the cytoplasm are common, and in several places there are groups of large, closely apposed foamy cells with small pyknotic nuclei. There are occasional giant binucleate or multinucleate cells, but none of the type found in benign synoviomas.

Clefts resembling synovial spaces are fairly numerous. Most of these are lined by flattened tumour cells and there is no pseudo-glandular arrangement. Neither mucus nor iron pigment is present, but in some of the necrotic foci there are a few cholesterol-crystal clefts.

The malignancy of the tumour is indicated by its great cellularity, cell pleomorphism, numerous mitoses and infiltrative growth. The structure is that of a malignant synovioma.

*Necropsy tissues. Lungs.* In the metastases, the tumour cells form diffuse areas completely replacing the pulmonary tissue or else are finely dispersed through surviving alveoli. In these less heavily infiltrated parts, mucus and tumour cells occupy the alveolar spaces. Though there is distinct resemblance between the diffuse parts of the pulmonary metastases and the primary growth, the cells in the lungs are less compactly arranged and show greater pleomorphism and mitotic activity. Being less closely packed, they are more distinct in outline and most of them are fusiform or elongated, with branching extremities which fuse with adjacent cells to form loose syncytial sheets (fig. 6). Running between these sheets are wide irregular clefts analogous to the smaller ones in the primary tumour.

In the areas of loose infiltration within alveoli, the tumour cells are very variable in shape, being fusiform, stellate, cuboidal, flattened or irregular. Abundant mucus is present in the alveoli (fig. 8) and the tumour cells lie free in it, fill the spaces as a loose reticulum or form an incomplete lining to the alveolar walls (figs. 7 and 8). Where the lining is a single layer of flattened or cuboidal cells, a glandular epithelial appearance is produced. Compact clusters of tumour cells (fig. 7) having a resemblance to swollen villous processes project into some of the alveoli. There is thus both a pseudo-acinar and

GROSS TUMOUR EMBOLISM OF AXILLARY ARTERY

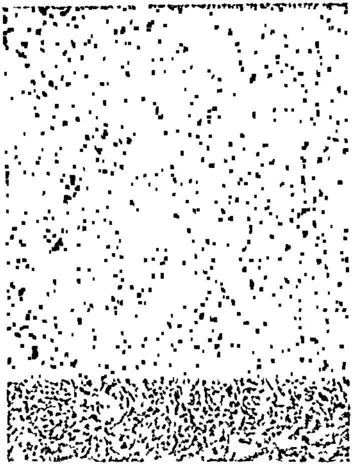

FIG. 4.—Primary tumour with synovial spaces. ×90.

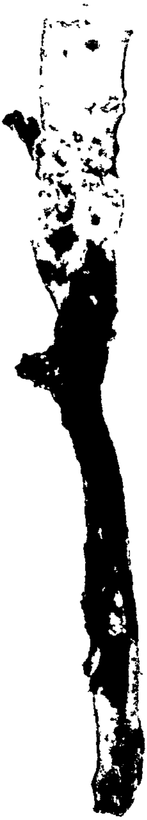

FIG. 3.—Left axillary artery occluded by tumour-thrombus. Natural size.

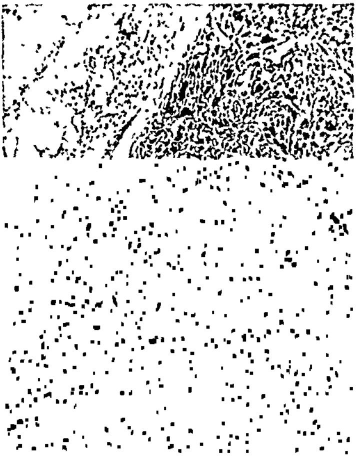

FIG. 5.—Primary tumour infiltrating subcutis. ×90.

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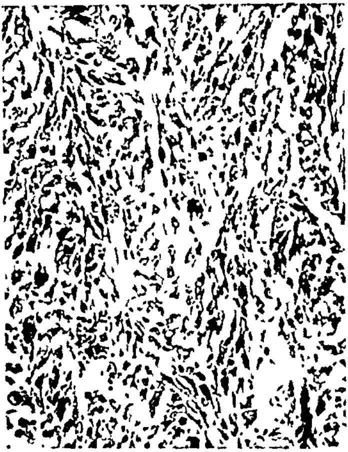

FIG. 6.—Pulmonary metastasis showing syncytial drifts of spindle cells. ×180.

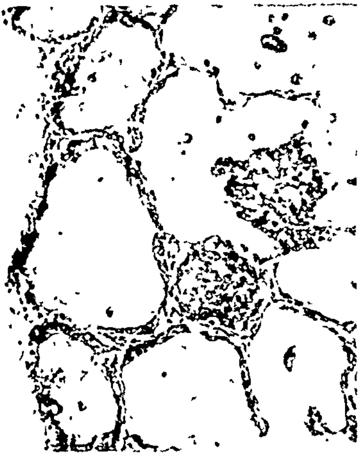

FIG. 7.—Pulmonary metastasis showing tumour cells lining the walls and projecting into alveoli. ×160.

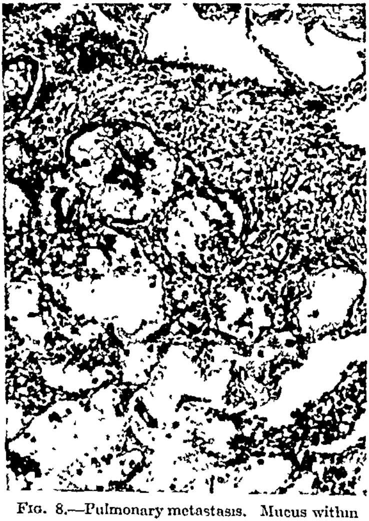

FIG. 8.—Pulmonary metastasis. Mucus within alveoli lined by tumour cells. ×100.

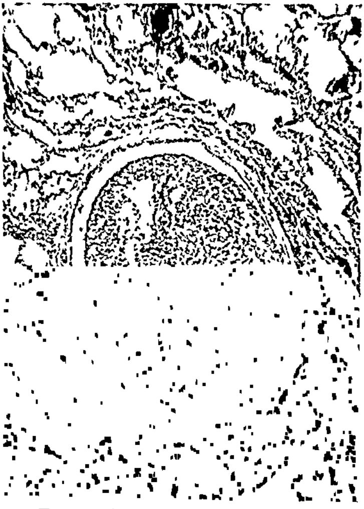

FIG. 9.—Pulmonary vein permeated by *growth.* ×30

pseudo-papillary arrangement in these areas. Where the tumour does not use the alveolar walls as a scaffold, stroma is scanty but fairly rich in capillary vessels. Little necrosis or hæmorrhage is present.

Invasion and permeation of tributary pulmonary veins are prominent. Growth within these vessels either fills the lumen as cellular tissue closely adherent to a large part of the wall (fig. 9) or is loose, richly mucinous and poorly cellular with groups of tumour cells situated mainly at the periphery of the lumen.

*Other organs.* Small tumour metastases of similar histological structure are present in the thyroid, lumbar vertebræ, myocardium of the left ventricle and leptomeninges overlying the parietal lobe of the left cerebrum, which is indented but not infiltrated. The other cerebral and cerebellar lesions are small hæmorrhagic infarcts with tumour-thrombus in adjacent arterioles. An embolus of tumour-thrombus also occupies the lumen of a small artery at the periphery of the thyroid deposit.

The clot which lay free in the main left pulmonary vein is composed of thrombus with very few tumour cells.

## Discussion

Arteries long resist tumour invasion, partly perhaps because of their compact musculo-elastic walls, and are rarely infiltrated by growth. Hence arterial tumour embolism due to penetration of systemic arterial walls is unlikely and indeed has never been recorded. Arterial tumour embolism follows direct invasion of the left chambers of the heart or of pulmonary veins, while passage of tumour cells through a patent foramen ovale is another possible route of entry for such cells into the arterial system.

Systemic dissemination of primary or secondary pulmonary neoplasms usually occurs through invasion of pulmonary veins, the emboli being in most cases of microscopic size. The rarity of emboli of macroscopic dimensions is indicated by the very few recorded instances.

In the case described here, not only was there sudden occlusion of a large artery by a large free tumour embolus but its source was clearly recognisable at necropsy, when extensive gross permeation of pulmonary veins was found. The histological structure of the growth resembles that of accepted cases of malignant synovioma and the circumscribed appearance of the primary tumour, which at operation was not visibly attached to the capsule of the knee joint, suggests that it may have been of tendon-sheath or bursal origin.

## Summary

Following permeation of tributary pulmonary veins by metastases of a malignant synovioma of the knee, gross arterial tumour embolism caused obstruction of the left axillary artery in a woman of 64.

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# SMOOTH-MUSCLE TUMOURS OF THE GASTRO-INTESTINAL TRACT AND RETROPERITONEAL TISSUES PRESENTING AS LARGE CYSTIC MASSES

George Lumb

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(Plates XX-XXVII)

Smooth-muscle tumours of the gastro-intestinal tract and retroperitoneal tissues have long been recognised. Neuber (1920) quotes Morgagni as being the first to describe a tumour of this kind in the stomach in 1762. Virchow (1863-67) suggested the subdivision of the intestinal cases into submucosal and subserosal, and a general account of the clinico-pathological relationships of this group was given by Bland-Sutton in 1893.

The chance finding of small sessile leiomyomata in the bowel wall *post mortem* is uncommon but well recognised. Golden and Stout (1941) had 23 in their autopsy series, an incidence of 0·39 per cent. Rieniets (1930) gives the much higher incidence of 16 per cent. in a series of 200 autopsies.

Cases producing clinical symptoms are also well established, but rare. There were 30 such in Golden and Stout's collected series covering the period 1913 to 1940 inclusive, while Marvin and Walters (1948) reviewed 16 malignant cases from the stomach alone, seen during the years 1907 to 1946, and point out that in the same period 10,000 cases of gastric carcinoma were seen in the same clinic, thus giving a good idea of the relative frequency of the condition.

Most of the tumours described are quite small and solid, arising either beneath the mucosa of the bowel and enlarging into the lumen, or producing a diffuse tumour in the muscular wall. A minority of intestinal cases, however, enlarge outwards into the peritoneal cavity—the so-called exo-enteric or subserous type, and these show a tendency to central liquefactive necrosis, so that they come to present as cystic tumours. In Golden and Stout's series, six cases of this exo-enteric—or subserous type—are recorded out of the 30 cases referred to above, and of these three were cystic. Marvin and Walters record only two examples of subserous tumours in their series, but it is not stated whether these were cystic.

The retroperitoneal tumours tend to grow to a larger size than the others and, as stated, they also may show cystic degeneration. This, however, is an unusual occurrence. Golden and Stout, for example, had only nine such cases in their series, which covered a period of 26½ years. In only one of these was the entire tumour available for *pathological study. In the remaining eight, one was an incidental* finding at operation and in seven, only biopsy specimens were obtained. A further example of a retroperitoneal smooth-muscle tumour was recorded by Willis in 1934.

Thus this group of tumours may be classified from the morbid anatomical point of view as follows :—

1. *Gastro-intestinal tract leiomyomas*

(*a*) *Submucous*—arising in the wall and extending as a tumour into the lumen of the bowel : this variety is nearly always of solid type.

(*b*) *Subserous exo-enteric*—arising in the wall and extending into the peritoneal space : these tumours are more commonly large and show a tendency to cystic degeneration.

(*c*) *Hour-glass*—a combination of (*a*) and (*b*) (fig. 5).

2. *Retroperitoneal leiomyomas*

Tumours arising from smooth muscle in the retroperitoneal tissues and showing a tendency to large size and cystic degeneration.

Type 1 (*a*) is the most common of these tumours. It may or may not be associated with mucosal ulceration. Types 1 (*b*), 1 (*c*) and 2 are all rare.

It is the purpose of the present paper to draw attention particularly to the relatively unusual group of cases which present as large cystic abdominal masses. A description is given of 7 recently observed examples, of which three arose from the stomach, three from the jejunum and one from the retroperitoneal tissues.

## CASE REPORTS

**Case 1.** A. B., a female aged 60, was seen on 12.3.35, complaining of frequency of micturition of three weeks' duration. On examination a large mass was found in the left upper abdomen. A barium meal showed some irregularity of outline of the lesser curvature near the pylorus, suggestive of its being stretched over a tumour. Laparotomy revealed a large tumour arising from the lesser curvature of the pyloric antrum and extending out into the lesser omentum. It was removed together with a small area of stomach wall surrounding its point of origin. There was no mucosal ulceration. The tumour was spherical in shape and 18·5 cm. in broadest diameter ; it presented a smooth outer surface covered by dilated vessels. On section it was found to be cystic, the cavity containing brownish fluid and necrotic debris. The inner aspect of the wall presented a shaggy appearance, being made up of irregular, brownish, partly necrotic tissue (fig. 1).

*Histology.* The tumour is composed principally of spindle cells with demonstrable myofibrils. There are many areas of degeneration and moderate numbers of mitotic figures.

*Subsequent history.* The patient was discharged fit on 4.5.35 and had no further abdominal symptoms of any sort. In 1946, 11 years later, at the age of 71, she had a cerebral thrombosis from which she recovered, but died on 12.7.49 from a second cerebral vascular catastrophe. Thus she survived her abdominal operation for 14 years and died, aged 74, as a result of a condition in no way referable to her abdominal tumour.

**Case 2.** L.B., a female aged 56, was seen in November 1932, complaining of pain in the back. A tumour was palpated in the abdomen. Laparotomy revealed a cystic mass, considered to be inoperable, in the epigastrium. A biopsy was taken and on histological examination a diagnosis of spindle-cell sarcoma was made. The patient continued in good health without loss of weight for one year, after which the mass increased in size. A second laparatomy was performed with a view to investigating the possibility of inserting radium seeds into the mass. At operation this was considered to be impracticable and the abdomen was closed. The patient developed bronchopneumonia post-operatively and died. At post-mortem examination on 12.2.34 a massive lobulated cystic tumour arising from the posterior wall of the mid-zone of the stomach was found (figs. 2 and 3). The mass extended upwards between the layers of the lesser omentum, then downwards behind the peritoneum with all the layers of the greater omentum lying anterior to it. Distended veins covered its surface, and on cutting it open a cystic cavity communicating with the lumen of the stomach was revealed (fig. 3). The cause of death was extensive bronchopneumonia and myocardial degeneration.

*Histology.* The tumour is made up of spindle cells of smooth-muscle origin which show a marked tendency to interlace. There are also many areas of undifferentiated cells. Mitotic figures are not a feature.

**Case 3.** W. N., a male aged 58, was seen on 6.6.49, complaining of colicky pains in the right lower abdominal quadrant of 9 weeks' duration. On examination he was in good health, but his waist circumference was 45 inches, although it was impossible to define any tumour boundaries. At laparotomy a huge cystic tumour was found arising from the anterior surface of the lesser curvature of the stomach and filling the abdomen from diaphragm to pelvis. It was growing between the two anterior layers of the greater omentum and was attached to the diaphragm and adjoining mesentery (fig. 4). It was removed, together with a portion of stomach measuring 4 × 3 cm. There was an area of mucosal ulceration in relation to the tumour, and a probe passed into the crater communicated with the central necrotic cavity of the mass. The specimen measured 42·5 cm. in long diameter and contained 7 litres of brownish fluid. Its outer surface was smooth except where attachments had been divided. On section the wall was thin and rather friable, and there were numerous shaggy necrotic projections extending into the central cavity.

*Histology.* The tumour is composed of spindle cells with myofibrils and numerous mitotic figures. Many giant forms and bizarre cell patterns are also seen. There is a tendency to " regimentation " of nuclei and considerable cell degeneration has occurred.

*Subsequent history.* The patient was discharged fit and well 11 days after operation, with a waist circumference of 34 inches. Three months later he returned with abdominal distension and at laparotomy multiple peritoneal metastases were found, scattered throughout the abdominal cavity, with a histological picture identical with that of the original tumour. No blood-borne metastases were discovered. The patient's general health deteriorated rapidly and he died as a result of bronchopneumonia and intestinal obstruction. There was no autopsy.

**Case 4.** F. P., a female aged 65, was seen on 4.1.49 complaining of abdominal swelling and loss of weight for one year, with abdominal pain of sudden onset, constipation and vomiting for 24 hours. On examination the abdomen was

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*Histology.* The tumour is composed principally of spindle cells with demonstrable myofibrils. There are many areas of degeneration and moderate numbers of mitotic figures.

this period. On examination no abnormalities were discovered other than a round tense mobile swelling in the left iliac fossa, which was hyper-resonant on percussion. Plain X-ray examination of the abdomen indicated that the mass contained air. At laparotomy a large mass was found arising from a loop of jejunum. It was firmly aderent anteriorly to the posterior wall of the bladder and laterally had invaded the wall of an adjoining loop of bowel. The surface of the tumour was lobulated and covered by distended veins. On section an "hour-glass" tumour was revealed (fig. 6), with a small portion projecting into the lumen of the bowel and a large cystic tumour, the cavity of which communicated with the jejunum at its point of origin, passing between the layers of the mesentery. The inner aspect of the cystic portion was shaggy and its contents consisted of necrotic debris and foul-smelling yellowish-brown fluid.

*Histology.* A spindle-cell tumour of smooth-muscle origin, with considerable cellular variation. Mitotic figures are numerous, and there is infiltration of the serous and muscular coats of the adjacent loop of jejunum.

*Subsequent history.* The patient made an uneventful recovery and was discharged fit and well and putting on weight. When last seen on 8.8.50 he was symptom-free.

**Case 6.** A. H., a female aged 45, was seen in 1937, with vague abdominal pain. On examination a tumour could be palpated in the right hypochondrium. Laparotomy revealed a tumour arising from a loop of jejunum. Both tumour and loop of bowel were resected. The specimen showed a cystic tumour arising in the wall of the jejunum and enlarging outwards between the layers of the mesentery (fig. 7). A point of communication between the bowel lumen and the necrotic centre of the mass was demonstrated (fig. 8), but no "hour-glass" formation was evident. The outer surface of the mass was covered with distended veins and section showed the central cavity to be lined by shaggy necrotic material.

*Histology.* The tumour is composed of interlacing smooth-muscle fibres. Two days after operation the patient collapsed and died. At autopsy, the cause of death was found to be hæmorrhage from the operation site. There were no metastases and no other evidence of disease.

**Case 7.** W. J. S., a male aged 36, complained of a painless abdominal swelling first noticed four years previously. Laparotomy revealed a large cystic retroperitoneal tumour arising from the pelvis and adherent to the sacrum. It was stripped off the sacrum and removed. The specimen was roughly spherical, measuring 16 cm. in diameter. It had a smooth outer surface and a firm wall 1·5 cm. thick. It contained brownish fluid and necrotic debris, and the inner surface, although smoother than in the other specimens in this series, showed friable brownish projections of the type so characteristic of this group of tumours.

*Histology.* The tumour is composed of spindle cells of smooth-muscle type, with some fibrous tissue substrate. Mitotic figures, giant cells and areas of degeneration and hæmorrhage are seen.

*Subsequent history.* The patient was discharged fit and well and when last seen on 6.8.50 was still free from symptoms.

To summarise, therefore, seven cases of smooth-muscle tumour are described which presented simply as large abdominal swellings without localising signs or symptoms. Each was surgically removable, although certain operative difficulties were experienced. There was no evidence of metastatic spread except in case 3, and in case 5 there was infiltration by the tumour into a neighbouring loop of bowel.

## DISCUSSION

*Macroscopic appearances.* As has already been said, the majority of the smooth-muscle tumours which arise in the gastro-intestinal tract, and especially those found at autopsy, consist of single, small firm masses. Multiple growths are rare (Marvin and Walters). The subserous group and those arising in the retroperitoneal tissues differ from this description in that they tend to grow to a large size and to undergo central cystic change owing to colliquative necrosis.

The wall of the tumour varies in thickness according to the degree of central necrosis, becoming very thin and friable when this process is advanced. Peritoneal metastasis may occur from such a tumour, following rupture (Holta, 1943 ; and see case 3).

The outer surface of the mass is smooth and lobulated (fig. 2) and frequently covered by numerous distended blood vessels. There is a notable tendency for attachments to form between the outer wall of the tumour, which is lying retroperitoneally (fig. 4), and either the mesentery or adjacent organs (cases 3-6). It is interesting to note that the rare occurrence of attachment to lumbo-sacral vertebræ by a retroperitoneal tumour was seen in case 7. In a search of the literature this feature was found recorded on two occasions only (Magoun, 1919 ; Golden and Stout, 1941, case 29).

The inner surface of the wall of these tumours is characteristically rough and shaggy, the irregular appearance being due to numerous projections of friable brownish necrotic tumour tissue (figs. 1 and 3).

The central cavity contains varying quantities of brownish fluid made up of liquefied necrotic material and altered blood. The amount of fluid may be large—7 litres (1½ gallons) in case 3. In cases 4 and 7, the cysts had to be partially evacuated at operation before it became possible to remove them.

*Site of origin.* It seems certain that, in common with all other smooth-muscle tumours of the bowel, the group under discussion arises from the external muscular coat, while the retroperitoneal tumours originate in small scattered areas of unstriped muscle lying in the loose connective tissue of the retroperitoneal region.

In the bowel tumours here recorded, it was always possible to observe the muscularis mucosæ stretched over the mass, between it and the mucosa, except of course in areas where ulceration had occurred.

In cases 4 and 5, which produced " hour-glass " tumours of the jejunum, an interesting picture was observed. Here it was possible to find a layer of normal smooth muscle running between the submucous part of the growth and the large cystic subserous mass, while the histological picture of the two portions was identical. When sections were cut at different levels through the tumour, this layer of muscle was absent in one zone, and it seems probable that in each case the tumour commenced in the outer layers of external muscular

SMOOTH-MUSCLE TUMOURS OF INTESTINE

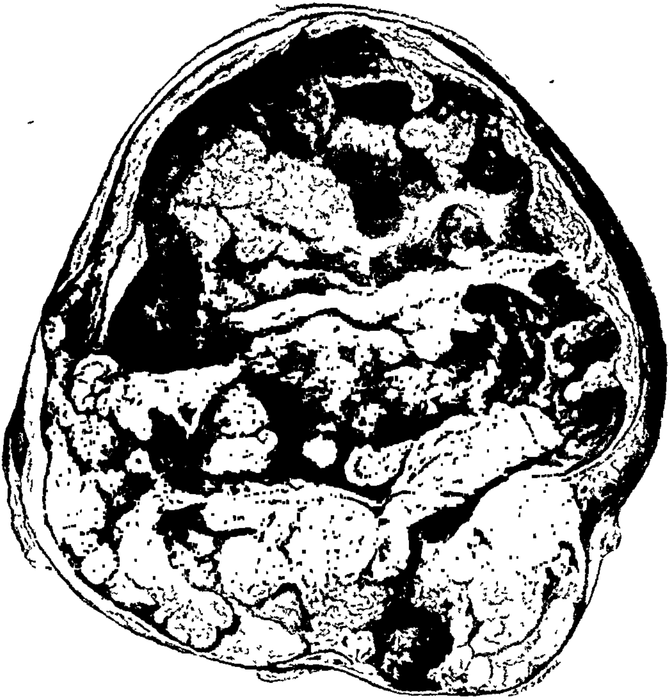

FIG. 1.—Drawing of inner aspect of cystic tumour; case 1. ×¾.

## Smooth muscle tumours of intestine

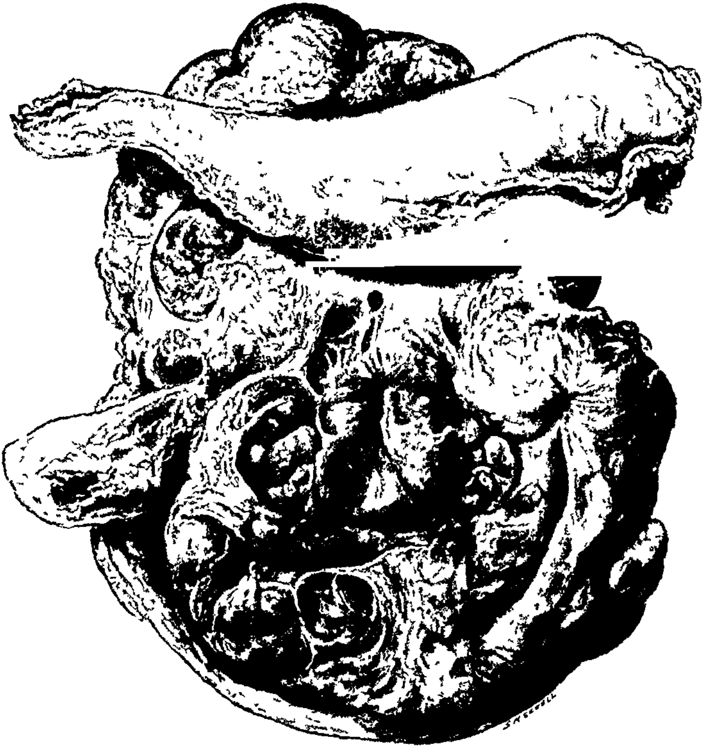

FIG 2 —Smooth and lobulated outer surface of tumour , case 2 ×c. ½

SMOOTH MUSCLE TUMOURS OF INTESTINE

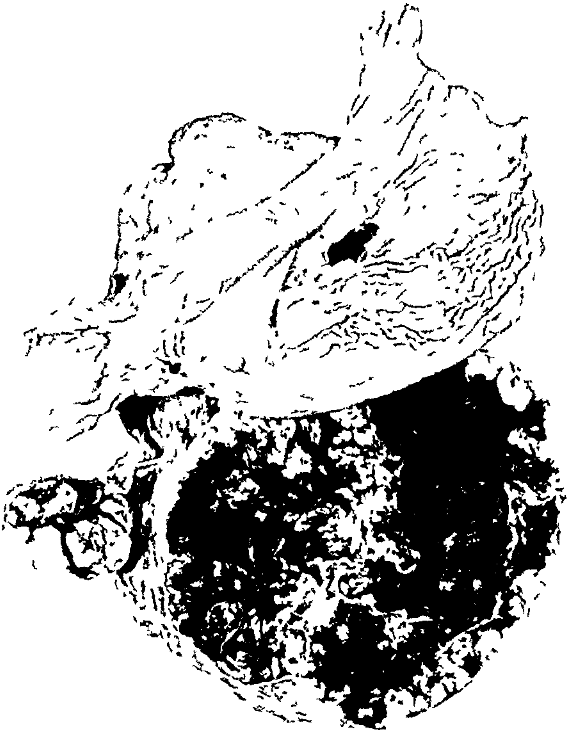

FIG 3—Cut surface of tumour from case 2, showing origin from stomach wall ×$\frac{2}{3}$

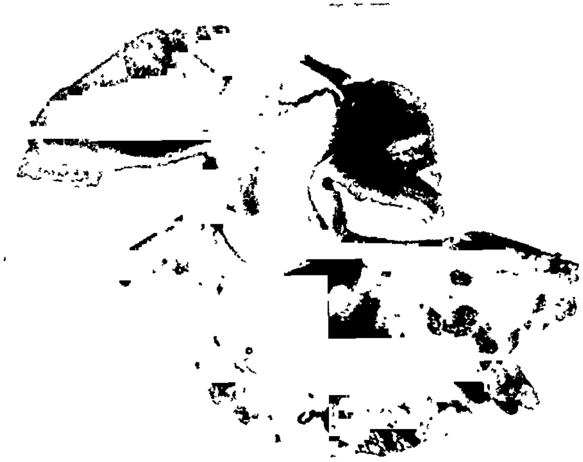

FIG. 5.—"Hour-glass" tumour from case 4, showing a small part of the jejunum in the upper part of the photograph, and part of the cyst wall in the lower half. ×1·3.

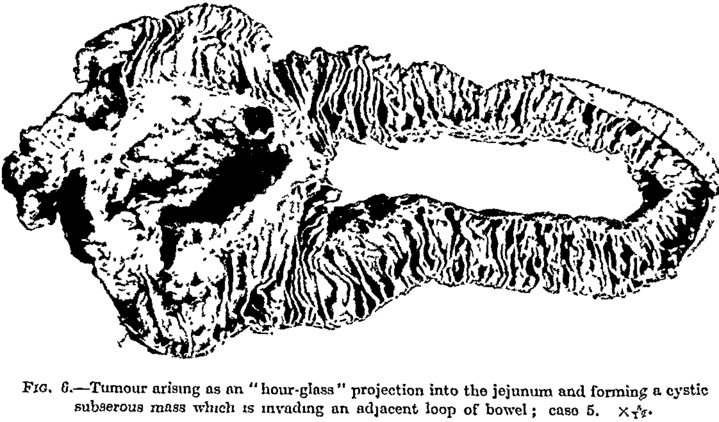

FIG. 6.—Tumour arising as an "hour-glass" projection into the jejunum and forming a cystic subserous mass which is invading an adjacent loop of bowel; case 5. $\times\frac{5}{12}$.

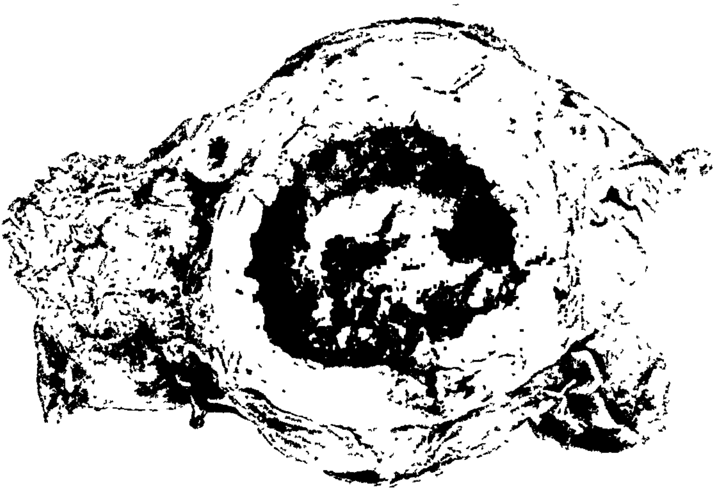

FIG. 7.—Cut surface of tumour from case 6, showing enlargement into the mesentery and necrotic cavitated interior. Part of the intestinal wall is seen on the right of the specimen. ×1·2.

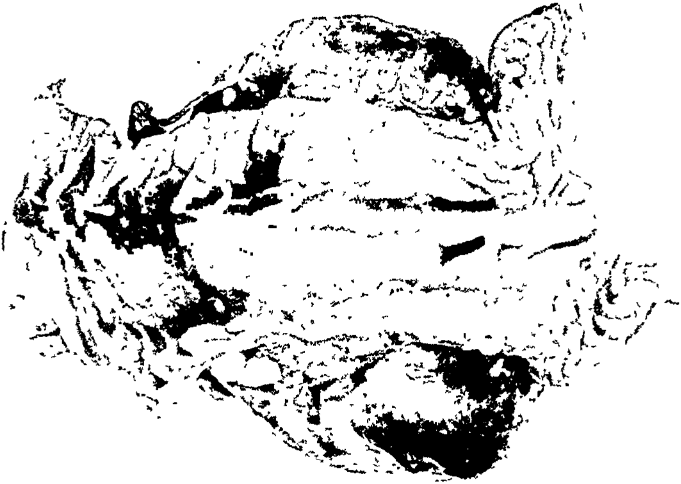

FIG. 8.—Mucosal surface of bowel to show communication with the necrotic interior of the tumour; case 6. ×1·25.

SMOOTH-MUSCLE TUMOURS OF INTESTINE

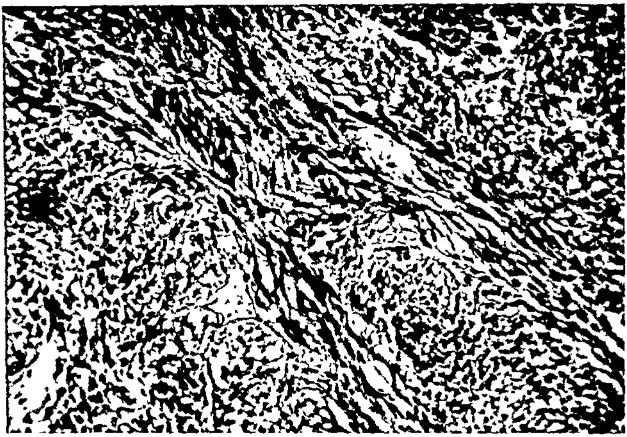

FIG. 9.—Spindle cells interlacing with each other and also with less well differentiated cells; case 2. Hæmatoxylin and eosin. ×150.

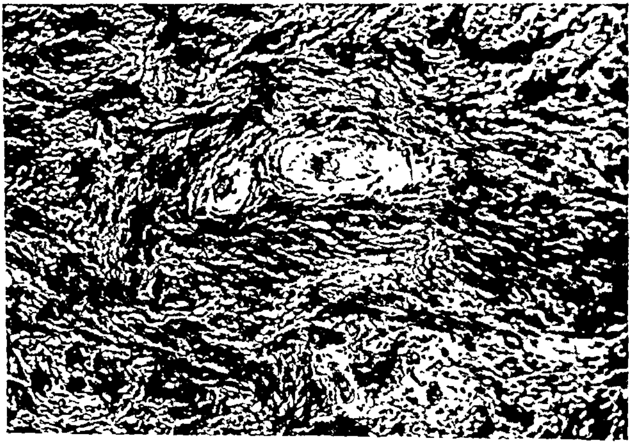

FIG. 10—Spindle cells with myofibrils arranged around hyalinised vessels; case 7. H. and E. ×150.

SMOOTH-MUSCLE TUMOURS OF INTESTINE

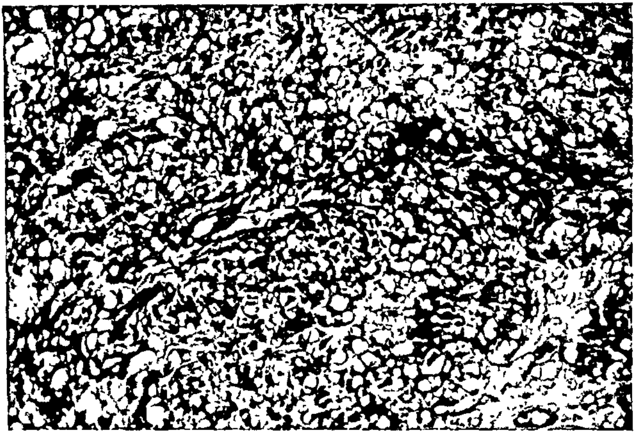

FIG. 11.—Lipoid degeneration of cytoplasm in an area of undifferentiated cells ; case 4. H. and E. ×150.

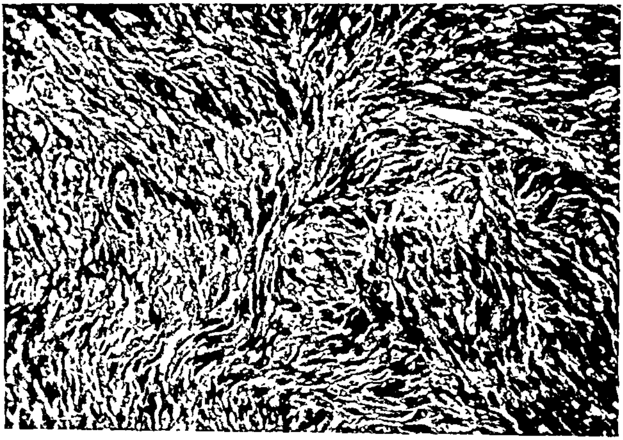

FIG. 12.—Regimentation of nuclei ; case 3. H. and E. ×150.

SMOOTH-MUSCLE TUMOURS OF INTESTINE

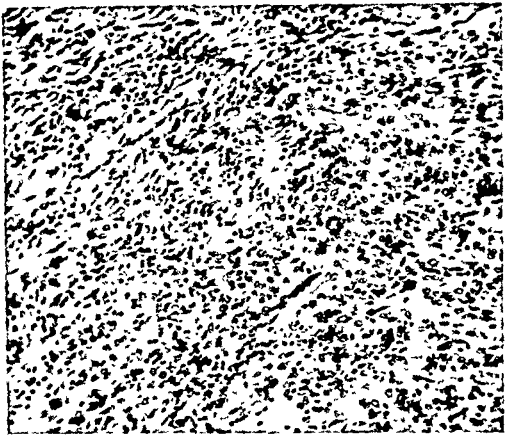

FIG. 13.—Area of undifferentiated cells; case 1. H. and E. ×150.

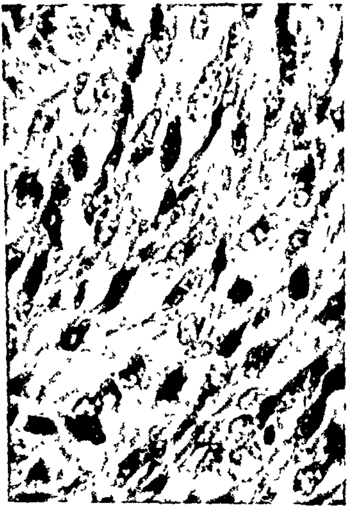

FIG. 14.—Mitotic figures in an area of active cell growth; case 3. H. and E. ×240.

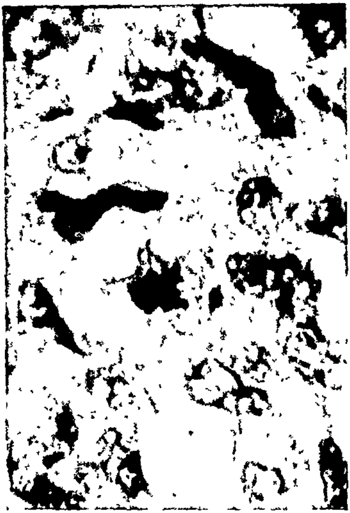

FIG. 15.—High-power view of mitotic figure in an area of undifferentiated cells; case 5. H. and E. ×800.

coat and burst through the inner layers only over a limited area to produce the submucosal tumour, thereby forming an even narrower neck to the " hour-glass " than was apparent macroscopically.

*Microscopic appearances.* The tumours are composed of bundles of spindle cells, sometimes interlacing (fig. 9), in which myofibrils can be demonstrated (fig. 10). Considerable variation in cell type is seen throughout all smooth-muscle tumours of the bowel, but in the subserous group, where the tendency to degeneration is greater, this variability is even more marked. Thus areas of degenerating cells undergoing fatty breakdown with formation of lipoid globules in the cytoplasm are frequent (fig. 11). Connective-tissue substrate is not a feature, but fibrous strands may be seen between the muscle-fibre groups, and in the degenerating portions there are often cellular myxoid areas.

There is a striking tendency for cells to align themselves so as to produce the appearance of nuclear " regimentation " or palisading (fig. 12). This fact, together with the degenerative changes described above, which in certain circumstances simulate those of the type-B tissue of the neurilemmoma, has caused doubt as to whether these tumours of bowel and retroperitoneal tissues arise in fact from smooth muscle or from neurilemma cells. The demonstration of myofibrils in the cells seems, however, to prove their origin, while the absence of a capsule between the tumour and the muscularis mucosæ is another feature in favour of a smooth-muscle origin, for a well-formed capsule is an almost constant feature of the neurilemmoma.

Most authors are now agreed that tumours of the type here described are of smooth-muscle origin, and Stout (1935; Golden and Stout, 1941) has even reclassified three tumours as leiomyomata which he had originally described under the title of neurilemmoma.

*Features of malignancy.* The question of microscopic evidence of malignancy has given rise to considerable discussion. It is a feature of the bowel tumours to show a gradual merging or interlacing of their cells with the normal muscle fibres of the muscularis mucosæ, a finding which might be taken to indicate infiltration and therefore malignancy and has been interpreted in this manner by some authors (Schindler *et al.*, 1946). However, it is almost always possible to demonstrate this phenomenon, even in the small intramural masses where no question of malignancy arises; it would seem wrong, therefore, to regard it as evidence of invasiveness.

Areas of undifferentiated cells are also found (fig. 13), but the tendency to degeneration described above gives rise to bizarre and apparently undifferentiated cell types, which may at first sight be thought malignant. In realisation of these difficulties most authors have tended to agree with the suggestion of Lemon and Broders (1942) that the finding of mitotic figures is sufficient proof of malignancy. Certainly if relatively large numbers of mitotic figures are found, these must be regarded as histological evidence of increased growth activity and as indicating the likelihood of malignancy (figs. 14 and 15). The converse, however, is not true, for, while it is considered that an occasional mitotic figure is no proof of malignancy, it must be admitted that among the rare examples of metastasis of smooth-muscle bowel tumours are cases where the primary growth was completely differentiated (Hunt, 1923; Demel, 1926; Melnick, 1932).

Thus bizarre cell patterns and apparent invasiveness are not necessarily indicative of malignancy, while on the other hand rare examples of metastasis from completely differentiated tumours are recorded. For these reasons only a general impression can be formed and a guarded prognosis given on histological grounds, and in view of the fact that examples of metastasis are rare, it is felt that the recommendation supported by Golden and Stout, to abandon the term leiomyosarcoma with its suggestion of clear-cut malignancy, is sound; and it is proposed to describe these tumours simply as leiomyomata, histologically either well or poorly differentiated. Thus all seven of the cases here described fall into the group of leiomyoma histologically poorly differentiated, in view of their anaplastic cellular make-up, active cellular division as evidenced by mitotic figures, very large size with central necrotic breakdown and tendency to invade surrounding structures. Metastasis, however, occurred only in case 3, where there was extensive peritoneal spread. In case 1 the patient lived for 14 years after operation without further symptoms, before dying of a cerebral vascular catastrophe at the age of 74.

*Metastasis.* The incidence of metastasis is somewhat difficult to assess. Golden and Stout give the figure of 30 per cent. for malignant cases while Pack and McNeer (1935) put it at 33 per cent. On the other hand, Marvin and Walters give 12 per cent. and Schindler *et al.* 15 per cent.

It seems clear that this apparent discrepancy arises out of the difficulty of histological criteria of malignancy. Thus the higher an author puts his proportion of malignant cases, the lower will be the percentage showing metastasis. It may be of some value therefore to quote actual figures for metastasis in all cases of unstriped-muscle tumours of the gut, without any attempt at subdivision into benign and malignant. Thus in Golden and Stout's series of 51 cases from the gastro-intestinal tract, there were three which showed metastasis, while of the 9 cases of retroperitoneal tumours reported by these authors, three showed metastasis. The value of this latter figure, however, is somewhat vitiated by the fact that in only one of these cases was the whole tumour available for study, and also that this was the only case on which an autopsy was performed.

It may be said, therefore, that metastasis is a rare event, but that it does occur from time to time; hence generalisation on the subject of prognosis is difficult if not impossible, and each case must be followed up carefully and treated on its merits.

## SUMMARY

1. Seven cases of large cystic smooth-muscle tumours of the gastro-intestinal tract (6 cases) and retroperitoneal tissues (1 case) are presented.

2. In every instance the complete absence of symptoms directly referable to the tumour is noted, and the bizarre character of the clinical picture is stressed.

3. The difficulty of assessing possible malignancy by histological means is discussed.

4. Prognosis based on the histology of these tumours must be guarded, and careful follow-up of all cases is not only in the best interest of the individual patient, but may also serve to throw light on the difficult question of the criteria of malignancy in general.

My grateful thanks are due to Prof. R. J. V. Pulvertaft for his encouragement, to Sir Ernest Rock Carling, Sir Stanford Cade and Mr G. T. Mullaly for making available the clinical records of the cases under their care, and to Dr Peter Hansell of the department of clinical photography, Westminster Hospital.

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612 . 112 . 3 . 083

# A METHOD FOR OBTAINING ACCURATE AND REPRODUCIBLE RESULTS IN EXPERIMENTS ON PHAGOCYTOSIS

G. E. Davies

*From the Biological Laboratories of Imperial Chemical Industries Limited, Hexagon House, Blackley, Manchester*

The opsonic technique has been largely abandoned in recent years, mainly because of the difficulty of obtaining reproducible quantitative results. Most authors have mentioned this difficulty: thus, Gordon and Atkin (1938) wrote (p. 206) " . . . phagocytosis experiments do not lend themselves to definite quantitative measurements . . .". In consequence, Gordon and Atkin contented themselves with demonstrating gross effects. For the work we had in mind, namely a study of the action of chemical compounds on phagocytosis of staphylococci by human leucocytes, the mere demonstration of gross effects was useless. By the technique described in this paper, however, we have been able to obtain reproducible quantitative results. Since by slight modifications it can be adapted to the study of many other problems connected with phagocytosis, we feel that it may be of some interest.

## Materials and methods

### *Bacterial suspension*

The aim was to obtain a suspension of bacteria which would, as far as possible, consist of individual cells in constant numbers from day to day. This was achieved as follows:—

The afternoon before a test, 0 5 ml of a 24-hour broth culture of the organism is pipetted on to the surface of 5 ml. of sterile nutrient agar contained in a 50 ml conical Pyrex flask After tilting to allow the liquid to spread evenly over the surface of the agar, the flask is incubated at 37° C On the following morning, 10 ml. of sterile saline and about 200 glass beads 2 mm. in diameter are added and the growth suspended and homogenised by rotating the flask by hand for 5 minutes. With any particular strain of organism the results of viable counts on suspensions prepared in this manner are fairly constant from day to day. The viable counts obtained with a strain of *Staphylococcus aureus* on six occasions are shown in table I. Table II shows that 85 97 per cent of the cells occur singly or in pairs as shown by stained films Some experiments carried out using autoclaved suspensions of bacteria were unsatisfactory, in that autoclaving caused the cells to clump and to stain less readily.

### *Preparation of leucocytes*

After a series of preliminary experiments, using animal leucocytes obtained in various ways, it was found that human leucocytes were preferable since they are bigger and stain better, and are more easily recognisable than those of the mouse, guinea-pig or rabbit. To avoid agglomeration of leucocytes caused by the clotting of plasma in the presence of coagulase-producing staphylococci, we used defibrinated blood collected in the apparatus shown in fig. 1. This consists of a small glass tube fitted with a siphon and U-shaped glass stirring-rod, one arm of which protrudes well above the top of the tube.

Table I

*Reproducibility of viable counts of Staph. " Dodd " from day to day*

| Date of expt. | No. of viable bacteria per ml. |
|---|---|
| 4.5.49 | 3,510,000 |
| 5.5.49 | 2,595,000 |
| 6.5.49 | 3,460,000 |
| 27.5.49 | 3,460,000 |
| 1.6.49 | 4,590,000 |
| 4.6.49 | 2,500,000 |

Table II

*Size of bacterial aggregates after suspensions had been rotated with glass beads (approximately 200 groups of cells counted)*

| No. of cells per group | Percentage distribution of groups in successive experiments | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Staph. (Dodd) | | | | | | Staph. I | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 |
| 1 | 41·5 | 45·5 | 43·5 | 44·5 | 46·5 | 46·5 | 36·0 | 30·5 | 34·5 | 32·0 | 39·0 |
| 2 | 55·5 | 50·5 | 50·5 | 49·5 | 47·5 | 47·0 | 57·5 | 62·0 | 55·0 | 53·0 | 48·0 |
| 3 | 1·5 | 2·0 | 4·0 | 4·0 | 5·5 | 5·5 | 2·5 | 2·5 | 5·0 | 6·5 | 4·5 |
| 4 | 1·0 | 1·5 | 1·0 | 1·5 | 0·5 | 1·0 | 2·5 | 3·5 | 4·5 | 5·0 | 5·5 |
| 5 | ... | 0·5 | 0·5 | ... | ... | ... | 0·5 | 1·0 | 0·5 | ... | 1·0 |
| 6 | 0·5 | ... | ... | ... | ... | ... | 0·5 | ... | ... | 1·0 | 0·5 |
| 7 | ... | ... | ... | ... | ... | ... | ... | ... | ... | 0·5 | 0·5 |
| 8 | ... | ... | ... | ... | ... | ... | ... | ... | ... | 0·5 | 0·5 |
| 9 | ... | ... | 0·5 | ... | ... | ... | ... | ... | 0·5 | 0·5 | ... |
| 10 | ... | ... | ... | ... | ... | ... | 0·5 | ... | ... | ... | ... |
| 11 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | 0·5 |
| 12 | ... | ... | ... | 0·5 | ... | ... | ... | ... | ... | ... | ... |
| 13 | ... | ... | ... | ... | ... | ... | ... | 0·5 | ... | ... | ... |
| 14 | ... | ... | ... | ... | ... | ... | ... | ... | ... | 1·0 | ... |

Blood is obtained by means of Wright's " sliver-pricker " (Wright, 1941-42). The drop of blood having been obtained, the siphon is applied to it and sufficient blood allowed to flow into the tube. The siphon is then withdrawn and the stirrer twirled between the finger and thumb until defibrination is complete; not less than 2 minutes should be allowed for this to happen. The start of defibrination is shown by an increased frothing of the blood.

### *Preparation of mixture of leucocytes and bacteria*

Capillary tubes as recommended by Wright and Colebrook (1921) were chosen in preference to other types of tubes because they require only small amounts of blood. Tubes internally coated with paraffin-wax are used to avoid loss of leucocytes caused by their adherence to the walls of the tube.

To prepare the mixture of blood and bacteria a rubber teat is fitted to the end of a Pasteur pipette internally coated with paraffin wax, and a mark made with grease pencil at a point about ¾ in from the distal end of the capillary. Defibrinated blood is drawn up to this mark and then a bubble of air allowed to enter A second volume of blood and one volume of bacteria, suspended either in saline or in a solution in saline of the compound being tested, are also introduced, each portion being separated from the next by a bubble of air. The contents of the pipette are then expelled on to a microscope slide coated with paraffin wax and mixed by drawing them into and expelling them from the pipette five times Finally the liquid is drawn well up into the capillary and the tip of the latter sealed in the pilot flame of a Bunsen Control experiments showed that no measurable phagocytosis happened during preparation of the tubes

### *Incubation of the mixtures*

The temperature of incubation (37° C) being an obvious choice, it remained for us to consider whether the tubes should remain stationary or be rotated Rotation results in a greater amount of phagocytosis but with staphylococci under the conditions described, adequate phagocytosis occurs if the tubes are left untouched for 30 minutes

### *Preparation of films and staining*

The sealed end of the capillary is broken off, a rubber teat fitted to the barrel and the contents mixed on a paraffined slide as before Drops of the mixture are then placed on clean microscope slides and films spread with the aid of the concave edged spreader described by Wright and Colebrook A rapid pushing action is used when spreading in order to obtain a film of suitable thickness The films are rapidly dried in air, fixed in methanol for 2 minutes and stained for 45 minutes in 1 in 50 Giemsa $\frac{1}{5}$ stain (Gurr's Improved Giemsa Stain R 66) in *p*H 7 buffer They are then washed in buffer solution and allowed to dry without blotting This method of spreading and staining results in large, well stained leucocytes Bacteria are easily differentiated from leucocytic granules by their deeper blue stain As many as 80 cocci have been counted in one leucocyte

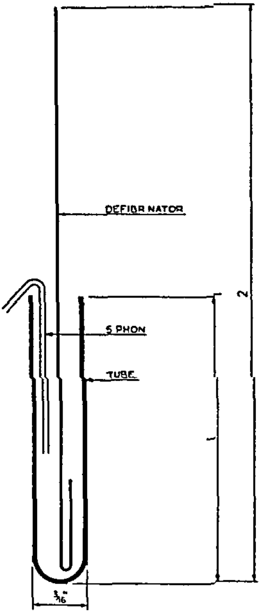

FIG 1 —Apparatus for collecting and defibrinating small quantities of blood ×2

### *Estimation of the amount of phagocytosis*

The slides are examined with a 1/12 in oil immersion lens Fifty neutrophilic polymorphs are observed and the number of bacteria present in each is recorded Although neutrophils were the only cells used in the investigation, eosinophils were frequently seen to contain bacteria number than would be expected from purely chance causes. This indicates either that leucocytes vary considerably in their ability to engulf bacteria, that the bacteria tend to agglutinate, or that when a leucocyte has engulfed a bacterium its ability to engulf other bacteria is increased thereby, or to some combination of these causes.

TABLE VI

*Means and variances of the results of typical experiments with the bacteria suspended in saline*

| Blood | Bacteria | No. of slides | Mean no. of bacteria per leucocyte | Variance | Coefficient of variation |  |
|---|---|---|---|---|---|---|
|  |  |  |  |  | per leucocyte (per cent.) | of mean per slide (per cent.) |
| GED | Staph. I | 2 | 13·3 | 64·1 | 60 | 8·5 |
| BYB | ,, | 3 | 20·6 | 70·4 | 41 | 5·8 |
| GED | ,, | 3 | 18·8 | 204·9 | 76 | 10·7 |
| GED | Staph. B | 3 | 14·0 | 44·9 | 48 | 6·8 |
| BYB | ,, | 3 | 9·5 | 55·9 | 79 | 11·2 |
| GED | Staph. E | 4 | 14·2 | 86·7 | 66 | 9·3 |
| BYB | ,, | 4 | 10·3 | 70·6 | 82 | 11·6 |

Large variations in both the mean and the variance occur from one control experiment to another. When the standard deviation is plotted against the mean for each slide in the whole of the data, it is seen that the standard deviation tends to increase linearly with increases in the mean (fig. 2). This indicates that the coefficient of variation (that is, the standard deviation expressed as a percentage of the mean) is a more appropriate measure of the variation than the standard deviation. Selected values of the coefficient of variation are included in table VI together with the coefficient of variation of the mean per slide, which is the former value divided by $\sqrt{50}$, 50 being the number of leucocytes counted per slide.

Examination of a large number of tests in this laboratory shows that there is no evidence of real variability between slides from the same capillary tube and between tubes treated alike in the same experiment. It does not necessarily follow that this is universally applicable and it is recommended that two tubes and two slides per tube be taken for each drug and each control: this represents the normal experiment in this laboratory. Such data are readily amenable to the "Analysis of Variance." The coefficient of variation of the mean per slide varies from about 5 to 12 per cent. but is usually between 7 and 9 per cent. in the experiments carried out in this laboratory. This is used as a guide to the order of accuracy that can be achieved. Other laboratories may obtain different values for the coefficient of variation, but if the experiment is carried out as recommended above, a valid estimate of the experimental error may be obtained from each experiment.

If $v$ per cent. is the coefficient of variation of the mean per slide, the coefficient of variation of the mean over the two tubes, with two slides per tube is $v/2$ per cent. since in our experiments there was no evidence of variation caused by the preparation of the tubes or the slides. From this we may readily derive the probable limits of error of the phagocytic coefficient, which is defined as :—

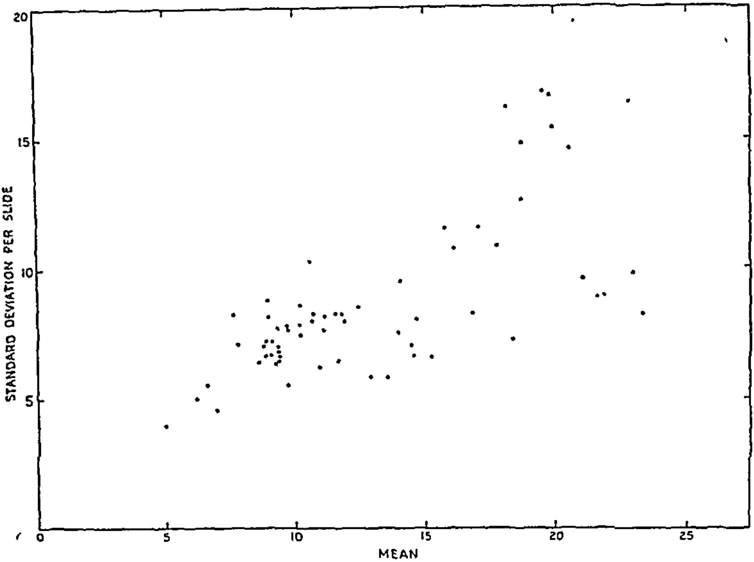

Fig. 2.

$$\frac{\text{Average no. of bacteria ingested by one polymorph in test solution.}}{\text{Average no. of bacteria ingested by one polymorph in saline.}}$$

Table VII

*95 per cent. limits of error of the phagocytic coefficient*

| Set-up | | Coefficient of variation of mean per slide = $v$ per cent. | | |
|---|---|---|---|---|
| | | 5 per cent. | 8 per cent. | 12 per cent. |
| One tube; 2 slides per tube | Upper limit $(1+2v/100)$ . . | 1·10 | 1·16 | 1·24 |
| | Lower limit $1/(1+2v/100)$ . . | 0·91 | 0·86 | 0·81 |
| 2 tubes; 2 slides per tube | Upper limit $(1+v\sqrt{2}/100)$ . . | 1·07 | 1·11 | 1·17 |
| | Lower limit $1/(1+v\sqrt{2}/100)$ . . | 0·93 | 0·90 | 0·85 |
| $m$ tubes; $n$ slides per tube | Upper limit $1+2v\sqrt{2}/100\sqrt{mn}$ . | ... | ... | ... |
| | Lower limit $1/(1+2v\sqrt{2}/100\sqrt{mn}$ . | ... | ... | ... |

number than would be expected from purely chance causes. This indicates either that leucocytes vary considerably in their ability to engulf bacteria, that the bacteria tend to agglutinate, or that when a leucocyte has engulfed a bacterium its ability to engulf other bacteria is increased thereby, or to some combination of these causes.

TABLE VI

*Means and variances of the results of typical experiments with the bacteria suspended in saline*

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|---|---|---|---|---|---|---|
| | | | | | per leucocyte (per cent.) | of mean per slide (per cent.) |
| GED | Staph. I | 2 | 13·3 | 64·1 | 60 | 8·5 |
| BYB | „ | 3 | 20·6 | 70·4 | 41 | 5·8 |
| GED | „ | 3 | 18·8 | 204·9 | 76 | 10·7 |
| GED | Staph. B | 3 | 14·0 | 44·9 | 48 | 6·8 |
| BYB | „ | 3 | 9·5 | 55·9 | 79 | 11·2 |
| GED | Staph. E | 4 | 14·2 | 86·7 | 66 | 9·3 |
| BYB | „ | 4 | 10·3 | 70·6 | 82 | 11·6 |

Large variations in both the mean and the variance occur from one control experiment to another. When the standard deviation is plotted against the mean for each slide in the whole of the data, it is seen that the standard deviation tends to increase linearly with increases in the mean (fig. 2). This indicates that the coefficient of variation (that is, the standard deviation expressed as a percentage of the mean) is a more appropriate measure of the variation than the standard deviation. Selected values of the coefficient of variation are included in table VI together with the coefficient of variation of the mean per slide, which is the former value divided by $\sqrt{50}$, 50 being the number of leucocytes counted per slide.

Examination of a large number of tests in this laboratory shows that there is no evidence of real variability between slides from the same capillary tube and between tubes treated alike in the same experiment. It does not necessarily follow that this is universally applicable and it is recommended that two tubes and two slides per tube be taken for each drug and each control: this represents the normal experiment in this laboratory. Such data are readily amenable to the "Analysis of Variance." The coefficient of variation of the mean per slide varies from about 5 to 12 per cent. but is usually between 7 and 9 per cent. in the experiments carried out in this laboratory. This is used as a guide to the order of accuracy that can be achieved. Other laboratories may obtain different values for the coefficient of variation, but if the experiment is carried out as recommended above, a valid estimate of the experimental error may be obtained from each experiment.

If $v$ per cent. is the coefficient of variation of the mean per slide,

# SHORT ARTICLES

616 . 329—018 . 7

## COLUMNAR CILIATED EPITHELIUM IN THE ADULT ŒSOPHAGUS

C Raeburn

*From the Central Histological Laboratory, Whittington Hospital, London*

(Plate XXVIII)

The œsophagus of the 2 mm embryo is lined by a single layer of cubical or columnar entodermal cells , beneath this a basal layer of cubical or spheroidal cells gradually forms and is complete at the 4 mm stage The superficial cells become more columnar, at 30 mm cilia appear, and by the 65 mm stage (eleven weeks) the entire œsophageal lining is ciliated Glycogen containing vesicular cells now appear in the basal layer , these gradually develop into squamous cells and, *pari passu* with their growth, the columnar cells atrophy and desquamate and the mature stratified squamous lining is completed about the 210 mm (twenty four weeks) stage (Maximow and Bloom, 1942 , Patton, 1946)

It would not be surprising if areas of columnar epithelium persisted, but the only recorded instance is that of a seven months' fœtus (Healey, 1919 20). It has not been reported hitherto in the adult

### *Case report*

A woman of 35 died after a brief hyperpyrexial illness thought to be fulminating meningococcal meningitis The positive autopsy findings were confined to the brain, pituitary and œsophagus

*Brain* The convolutions were flattened and a pronounced cerebello medullary pressure cone was present The entire ventricular system was dilated, particularly the left lateral ventricle, into which bulged a slightly nodular mass 3 cm in diameter, which on section was seen to arise in the tail of the caudate nucleus It contained small cysts and foci of calcification

*Pituitary* A discrete yellow nodule 0 3 cm in diameter was present in the anterior lobe and the pars intermedia contained a colloid cyst 0 2 cm in diameter

*Œsophagus* The lower half of the œsophageal mucosa exhibited irregular and elliptical, purple coloured and slightly recessed areas ranging from about 0 2 to 2 cm in diameter The surface of these areas had a velvety texture and at the time it was thought that they might be composed of gastric mucosa

### *Histology*

The cerebral neoplasm is a well differentiated astrocytic glioma The anterior lobe of the pituitary contains an area, mainly of chromophobe cells but with groups of eosinophil and a few basophil cells, which, though compact and well demarcated macroscopically, histologically merges with the surrounding glandular tissue and is not encapsulated It is a malformation rather than a true adenoma

The unusual appearance of the œsophagus is due to the presence of islands of ciliated columnar epithelium resting on a basal layer of ovoid cells with more densely staining nuclei and clear cytoplasm (fig.). Best's glycogen stain gives a negative result but Mayer's muci-carmine stain shows the presence of mucin droplets in both basal and columnar cells. The transition from squamous to columnar epithelium has been largely destroyed by post-mortem desquamation but fragments of a single layer of cubical epithelium remain at the margins of the ciliated plaques.

This œsophageal anomaly indicates a localised arrest of development and consequent persistence of the original embryonic entodermal epithelium.

My acknowledgments are due to Dr A. B. Bratton for helpful criticism and to Mr G. W. Moore for the photography.

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616 . 37—002 . 692—053 . 32

# GUMMA OF THE PANCREAS IN A PREMATURE INFANT

C. Raeburn

*From the Central Histological Laboratory, Whittington Hospital, London*

(Plates XXIX and XXX)

Congenital syphilitic fibrosis of the pancreas is still occasionally seen but a congenital gumma of that organ has always been exceedingly rare. Herxheimer (1908) cites three cases, two seen by von Klebs and Birch-Hirschfeld and one by Beck. Ghon (1936) mentions the possibility of gumma but gives no reference.

### *Clinical history*

During her first pregnancy a woman was found to have a strongly positive Wassermann reaction. She failed to receive treatment and spontaneous delivery occurred in the thirty-second week of pregnancy. The child had the typical skin lesions of pemphigus neonatorum and was immediately treated with penicillin. The bullæ healed remarkably well but the general condition of the infant deteriorated and it died on the seventh day.

### *Necropsy*

The body was that of an emaciated and slightly œdematous premature female infant, 2½ lb. in weight, with wizened facies and the flat, slightly crusted, dull red scars of healing pemphigus equally distributed over the entire body surface; there was approximately one scar 0·5 cm. in diameter, to each 25 sq. cm.

# CILIATED EPITHELIUM IN ADULT ŒSOPHAGUS

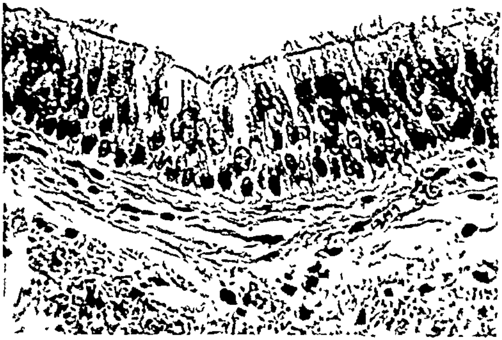

FIG.—Columnar ciliated epithelium in the adult œsophagus. Hæmatoxylin and eosin. ×400

of skin surface. The upper respiratory passages presented no abnormality but the lungs, which were perceptibly tough on section, were deeply congested and showed basal bronchopneumonia and small circumscribed areas of atelectasis. The foramen ovale and ductus arteriosus were widely patent. The cardia of the stomach showed patchy brown discolouration, mainly anteriorly and along the proximal third of the greater curvature; in these areas the wall was thickened and the mucosa finely granular. Similar thickened and discoloured patches occurred in the duodenum in the parts related to the pancreas. The head of the pancreas appeared as a hard, spherical, fibrous mass 2·5 cm. in diameter adherent to the duodenum. This on section proved to be a polycystic gumma with necrotic, semi-purulent, greenish white contents (fig. 1). The body and tail of the pancreas were fibrotic. The remaining organs were macroscopically normal.

## *Histology*

Portions of lung, stomach, duodenum, pancreas, liver, spleen, adrenal and kidney were embedded in paraffin and sections stained with hæmatoxylin and eosin, iron hæmatoxylin and Van Gieson, and Weigert's stain for elastic fibres. In addition portions of liver, adrenal, kidney and pancreas were treated by Levaditi's method for spirochætes.

All the organs examined are œdematous and active hæmopoiesis is present in the spleen. The specific lesions occur in some degree in all these tissues with the exception of the spleen and adrenal. The *pancreatic* lesion is a typical gummatous necrosis with pericellular fibrosis of the surviving glandular tissue. At the margin of the necrotic area the cellular reaction is more pleomorphic than in the average gumma, foci of neutrophil leucocytes being mingled with plasma cells, lymphocytes and occasional small multinucleated cells. The process is walled off by dense collagenous tissue in which are a few miliary gummata and scanty glandular elements (fig. 2). Numerous spirochætes are demonstrable in the periphery of the necrotic zone (fig. 3). The *duodenum* is involved by contiguity and miliary gummata are present in the submucosa, while the inflammatory cellular exudate permeates the mucosa itself, including the basal parts of the villi. In the fundal portion of the *stomach* the thickened areas contain many lymphocytes, plasma cells and fibroblasts, obliterative arteritis is a prominent feature and minute necroses and mucosal ulcers are present (fig. 4). The *lungs* show congestion, partial atelectasis and focal bronchopneumonia. There is diffuse, delicate fibrosis, generalised atrophy of elastic tissue and prominent arteritis (fig. 5). The *liver* changes are minimal, consisting only of slight fibrosis. Myeloid tissue is present along the portal tracts and there is focal erythropoiesis in the parenchyma. In the *kidney* there is endarteritis of occasional interlobular and arcuate arteries, and focal, delicate, reticular fibrosis.

## *Summary*

A case of congenital syphilis is described with the characteristic lesions in lung and kidney, the unusal feature of involvement of the stomach and duodenum and the exceedingly rare gumma of the pancreas.

My acknowledgments are due to Dr A. B. Bratton for helpful criticism and to Mr G. W. Moore for the photography.

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616 . 26—007 . 43—06 : 616 . 12—007 . 1

# A CARDIAC ABNORMALITY IN THE FŒTUS, APPARENTLY RESULTING FROM PREMATURE CLOSURE OF THE FORAMEN OVALE DUE TO PRESSURE OF A DIAPHRAGMATIC HERNIA

C. Raeburn

*From the Central Histological Laboratory, Whittington Hospital, London*

The individual malformations to be described are in themselves not of great rarity but the induction of a cardiac abnormality as a result of a primary diaphragmatic defect is possibly of importance in the study of fœtal pathology.

## *Case report*

The first child of a healthy mother aged 19 years, whose pregnancy and delivery were normal, failed to breathe and died within a few minutes.

*Necropsy.* The body was that of a full-term female infant weighing 6 lb. 13 oz. The chest was barrel-shaped and the abdomen slightly scaphoid. The umbilical cord was normal. The left side of the diaphragm was deficient and the thorax contained the stomach, spleen and intestines, while the anterior wall of the under-developed abdominal cavity rested against the vertebral column. The greatly enlarged heart was displaced to the right and the hypoplastic left lung was twisted upon its hilum. The thoracic cage and contained viscera, together with the thoracic and lumbar vertebræ and all organs down to and including the kidneys, were excised and fixed for detailed study.

*Thoracic structures.* The left crus and left cupola of the diaphragm were absent and the hiatus was occupied by a thin, transparent pleuroperitoneal membrane, with a crescentic, thickened anterior border. This membrane was invaginated by the abdominal viscera upwards to the left apex of the thoracic cavity and medially to the anterior border of the normally developed but atelectatic right lung. The lower œsophagus deviated to the right to a maximum of 2·5 cm. and the inverted stomach, greater curvature uppermost, lay above the left lobe of the liver and in contact with the anterior chest wall. The first part of the duodenum was flexed round the free border of the right crus of the diaphragm, the remainder occupying the normal position ; but the entire small intestine lay in the left half of the thoracic cavity and, behind it, in the paravertebral region, lay the cæcum and the ascending and transverse colon. The spleen had been drawn antero-medially by the displaced stomach and the attachment of the pleuroperitoneal membrane on the left side bisected the left adrenal, the upper half of which, demarcated by a well-defined groove, thus lay within the thoracic cavity. The left lung had failed to develop beyond a maximum length of 3·5 cm. and an average thickness of 0·5 cm. and the compressed lung tissue provided only the slightest cover for the bronchial structures, the edges of the lobes being transparent. This lung had, as a result of the pressure of the ascending gut, rotated upon its hilum so that the lower lobe passed backwards and upwards and the lung " apex " was formed by the true posterior margin of the base of the lower lobe compressed into the angle between the trachea and the subclavian vessels. The apex of the upper lobe had swung anteriorly and downwards and lay in contact with the upper part of the left border of the pericardium. This torsion through 180° had reduced the left pulmonary artery to a minute irregular slit. The lumina of the left pulmonary veins were similarly obstructed and the lateral wall of the left auricle was distorted and displaced medially, thus diminishing its cavity. The foramen ovale persisted as a crescentic valvular slit of small size. The cavity

## GUMMA OF PANCREAS IN AN INFANT

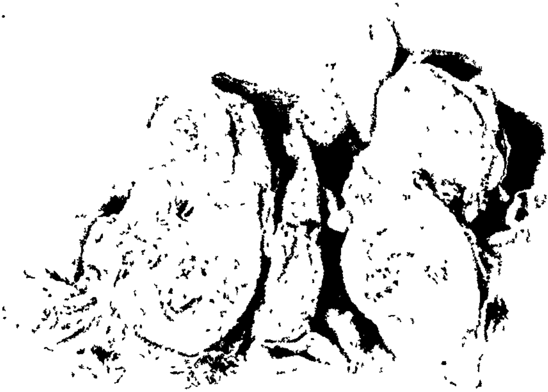

FIG. 1.—Macroscopic appearance of gumma in head of pancreas. ×1·5.

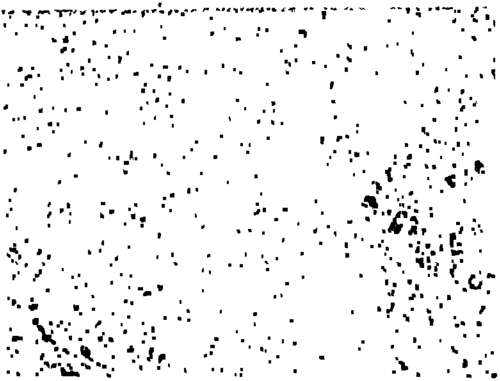

FIG. 2.—Gumma of pancreas showing extensive necrosis, fibrosis and a few remaining pancreatic tubules. Hæmatoxylin and eosin. ×70.

## GUMMA OF PANCREAS IN AN INFANT

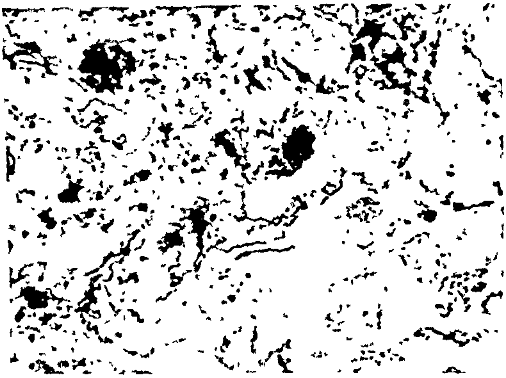

FIG. 3.—Spirochaetes in gumma of pancreas Levaditi. ×1300

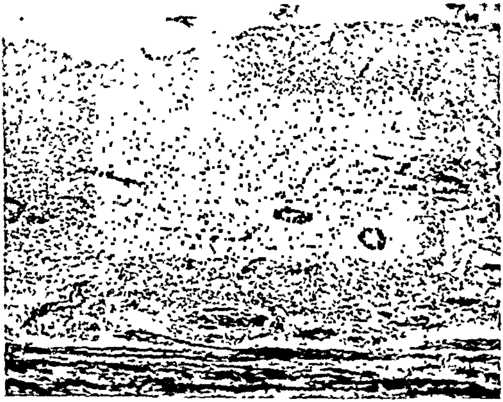

FIG. 4.—Syphilitic inflammation of stomach The muscularis mucosae is destroyed and obliterative arteritis and superficial necrosis (top left) are present H and E ×70

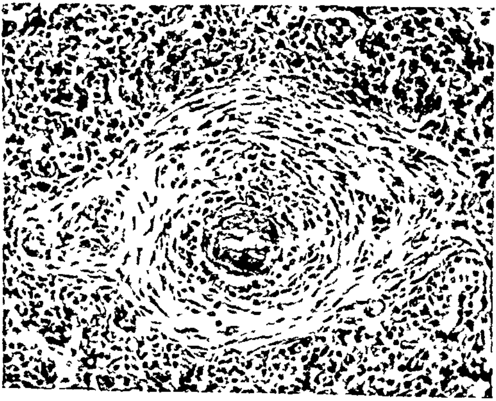

FIG. 5.—Obliterative arteritis affecting a pulmonary vessel H and E ×230.

of the left ventricle extended downwards as a rapidly diminishing cone, 3 cm. in length, from a mitral valve 1·5 cm. in circumference and an aortic valve 0·6 cm. in circumference. Around this hypoplastic left ventricle the myocardium averaged 0·4 cm. in thickness (fig.). The right ventricle formed the entire anterior surface and apex of the heart and was hypertrophied to a thickness of 0·4 cm. The tricuspid valve measured 3·6 cm. in circumference, the pulmonary valve 2·0 cm. The main pulmonary artery was dilated and hypertrophied and the diameter of the right pulmonary artery was 0·25 cm., that of the distorted left pulmonary artery not more than 0·1 cm. The ductus arteriosus diminished from 1·6 cm. in circumference at its origin to 1·1 cm. at its termination. The circumference of the aorta proximal to the ductus was 0·8 cm. and immediately distal to the ductus 1·4 cm. The pulmonary trunk and ductus formed a continuous artery from which the right and left pulmonary arteries arose as lateral branches (fig.).

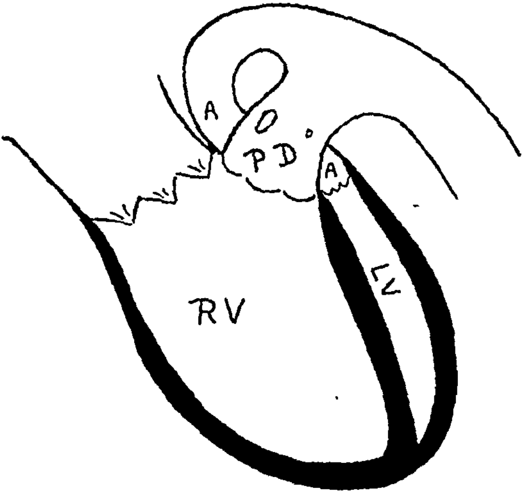

FIG.—Diagrammatic representation of the multiple defects of the heart. L.V. Hypoplastic left ventricle. A. Hypoplastic aorta. R.V. Enlarged right ventricle. P.D. Pulmonary artery-ductus arteriosus trunk showing the orifices of the pulmonary arteries: right, normal; left, minute.

## *Discussion*

Cardiac anomalies generally arise between the fifth and eighth weeks of embryonic life (6-20 mm.) when metamorphosis to the septate heart is occurring. In this instance the valves and septa are normal and the abnormalities to be explained are the hypoplasia of the left ventricle and aorta and the hypertrophy of the right ventricle, pulmonary artery and ductus arteriosus.

At the eighth week (20 mm.) the pleuroperitoneal membrane should close and separate the two body cavities and at the same time muscle fibres grow into the edge of the membrane from the body wall to form the future diaphragm.

When the membrane fails to close, the abdominal viscera herniate into the thorax but are not covered by membrane, and when the membrane closes, but muscle permeation is deficient, the result is a hernia covered, as in this case, by pleuroperitoneal membrane. The commencement of the series of events may therefore be placed at or about the ninth week.

In this case as the abdominal contents increased in size they followed the line of least resistance into the thorax and the abdominal cavity remained under-developed. On the left side a crescentic, thickened edge of membrane extended from above the left lobe of the liver round the antero-lateral wall of the thorax to the left adrenal, so that the greater part of the malformed area lay posterior to the transverse median longitudinal plane of the body cavities. Thus as the intestines rose into the thorax they first pushed against the posterior part of the base of the developing lung, carrying it upwards and backwards and so initiating the pulmonary rotation.

The configuration of the lung and the line of fission between the upper and lower lobes which is present at the 30-mm. (nine weeks) stage also predispose to backward rather than forward displacement of the lower lobe. The torsion of the left lung would constrict the left pulmonary artery and left pulmonary veins and the twist on the lateral wall of the left auricle distort and considerably diminish the capacity of the auricular cavity. As development progressed the intestines would pack tightly into the left side of the thorax and displace the mediastinum to the right. All the organs confined within the pleuroperitoneal membrane would act as one solid mass, with the main pressure exerted against the compressible structures on the medial side, particularly the left auricle, the walls of which must have been almost in apposition. The distortion of the left auricle is, it is suggested, the important cardiac deformity, for it led to an obstruction to the flow of blood from right auricle to left auricle through the foramen ovale. The left ventricle and proximal aorta, lacking the dynamic impulse of full distension, have failed to develop and conversely the hypertrophy of the right ventricle, pulmonary artery and ductus arteriosus is commensurate with the additional work thrown upon them by blockage of the foramen ovale and the acquisition of the bulk of the systemic circulation by the right ventricle. Functionally it is a bilocular heart.

The difference between fœtal and post-natal pathology is largely one of difficulty of observation and, though the case described is an example of a gross malformation inducing gross defects in a neighbouring structure, it may reasonably be suggested that a consideration of the mutually induced modifications of structure in developing organs is more likely to be of value than the attribution of anomalies to an unmeasurable defect of the germ plasm.

No relevant references have been traced.

## *Summary*

A multiple congenital anomaly of the heart comprising left ventricular and aortic hypoplasia, relative aortic stenosis, hypertrophy of the right ventricle, hypertrophy and dilatation of the pulmonary artery and hypertrophy of the ductus arteriosus, is considered to be due to functional closure of the foramen ovale by the pressure of a diaphragmatic hernia.

My thanks are due to Dr A. B. Bratton for helpful criticism.

576 . 809 . 72 : 576 . 851 . 42

# A NEW ANTIGEN OF *BRUCELLA ABORTUS*

H L Wolff and J E Dinger

*From the Laboratory for Tropical Hygiene of the State University, Leiden*

We recently received from Prof C D de Langen a strain of bacterium which was indistinguishable morphologically and biochemically from *Brucella abortus* This had been isolated from the duodenal contents of a patient who at one time had suffered from brucellosis The organism was agglutinated by the serum of the patient from whom it had been isolated, but not, or only to a slight extent, by the rabbit anti Brucella sera used by us for diagnostic purposes Besides this, the serum of the patient, though effective against his own organism, failed to agglutinate laboratory strains of Brucella

These rather paradoxical observations seemed to justify a thorough investigation of the strain

## *Experimental*

*Morphology and biochemical reactions* The organism was a straight, slender to stout, non motile, Gram negative, unencapsulated, ellipsoid rod, varying in length from 0 3 to 1 5 μ and not exceeding 0 3 μ in thickness Growth was improved by increase in the carbon dioxide concentration and in the humidity of the atmosphere , it was retarded by 1 30,000 thionin, but not by 1 25,000 basic fuchsin or 1 25,000 gentian violet , the colonies were smooth and looked rather moist No acid was formed in any of the usual laboratory sugars and no indol was formed in trypsin broth

In all these respects the strain, conveniently called L agreed closely with the strains of Brucella so far described

*Antigenic constitution* Agglutination tests were carried out as far as possible in parallel The tubes were kept at 37° C for two hours and then for a further 16 hours at room temperature (18° C ) before reading It made no difference whether agglutination titres were determined at 37° or at 50° C

Though the strain was not agglutinated by the rabbit anti Brucella sera kept in the laboratory for diagnostic purposes, it was consistently agglutinated by the sera of patients who were suffering or had suffered from brucellosis Agglutination was much slower than with typical Brucella strains and the flocculi were much smaller There appeared to be no correlation between the agglutinin titre of these sera against typical *Br abortus* strains and that against the L strain (table I)

Sera were then absorbed with typical *Br abortus* (strain 63) and the L strains as follows They were diluted to 1 in 25, mixed with the absorbing bacterial suspensions and incubated for two hours at 37° C At the end of a further 33 hours at 4° C the mixtures were centrifuged and the supernatants tested for their agglutination titre against *Br abortus* strain 63, *Br melitensis* and strain L Table II shows that strain L absorbs from human anti Brucella sera all Brucella agglutinins while typical *Br abortus* does not absorb all the L agglutinins It seems reasonable to conclude that strain L possesses, beside antigens A and M, a third antigen, L

Heating strain L for thirty minutes at 100° C greatly reduced the amount of L antigen, as judged by the reduced agglutinability of the heated organism with human anti Brucella sera After such treatment the L strain was readily agglutinated by rabbit anti Brucella sera (table III), presumably because the A and M antigens were now exposed

## *Discussion*

There is an obvious parallel between the antigen L of our Brucella strain and the Vi antigen of some strains of *Salmonella typhi*. Typhoid bacilli which possess the Vi antigen are readily agglutinated by the serum of typhoid carriers but when the Widal test is carried out with laboratory strains that have lost

TABLE I

*Comparison of agglutinin titres of rabbit anti-Brucella sera and of the sera of Brucella carriers against various Brucella strains*

| Sera | Strains | | | |
|---|---|---|---|---|
| | *Br. abortus* | L | *Br. melitensis* | *Br. abortus-suis* |
| Rabbit 237/5 . . . . | 1600 | 0 | 400 | 400 |
| „ 237/6 . . . . | 1600 | 0 | 200 | 1600 |
| „ 237/14 . . . . | 3200 | 0 | 800 | 800 |
| „ 579 . . . . | 800 | 0 | 50 | 200 |
| Patient I . . . . | 1600 | 3200 | 800 | 800 |
| „ IIa . . . . | 800 | 1600 | 200 | 200 |
| „ IIb . . . . | 800 | 1600 | 200 | 200 |
| „ IIc . . . . | 400 | 3200 | 200 | 100 |
| „ IId . . . . | 200 | 1600 | 200 | 100 |
| „ IIIa . . . . | 800 | 1600 | 200 | 400 |
| „ IIIb . . . . | 800 | 1600 | 200 | 200 |

TABLE II

*Agglutinin titres of sera before and after absorption with* Br. abortus *strain* 63 *and with strain L*

| Strain | Serum from patient | | | | | |
|---|---|---|---|---|---|---|
| | IIa | | | IIc | | |
| | absorbed with | | | absorbed with | | |
| | 0 | *Br. abortus*, strain 63 | strain L | 0 | *Br. abortus*, strain 63 | strain L |
| L . . . . . | 1600 | 400 | 0 | 3200 | 800 | 0 |
| *Br. abortus*, strain 63 . | 800 | 0 | 0 | 400 | 0 | 0 |
| *Br. melitensis* . . | 200 | 0 | 0 | 200 | 0 | 0 |

TABLE III

*Agglutinin titres of sera against strain L before and after heating*

| Serum | Organism used : strain L | | | | |
|---|---|---|---|---|---|
| | unheated | heated ½ hr. at 45° C. | heated ½ hr. at 56° C. | heated ½ hr. at 65° C. | heated ½ hr. at 100° C. |
| Patient IIIa . | 1600 | 400 | 400 | 400 | 400 |
| Rabbit 579 . | 0 | 0 | 0 | 0 | 400 |

the Vi antigen, or with "Ficker" suspensions without Vi, the sera always fail to agglutinate.

As species of Brucella possess no H antigen, the failure of laboratory sera that do not contain anti-L to agglutinate the L strain is evidently due to the fact that the L antigen completely covers the surface of the A and M antigens. The same phenomenon is seen in typhoid bacilli that possess the Vi antigen, but whose H antigen is either absent or badly developed. Primary isolation in such instances yields a strain that is not agglutinated by laboratory sera.

In both cases, therefore, we are dealing with antigens that are able to induce in the carrier the production of specific agglutinins, but envelop the bacteria and make them inagglutinable by laboratory sera prepared by the use of strains from which this particular antigen is absent. *So far strain L has retained* antigen L through numerous subcultures. If this should continue, and it is found that the sera of all Br. *abortus* carriers can agglutinate Brucella strains possessing the L antigen, the L strain might be of as great diagnostic value in the detection of *Br. abortus* carriers as the Vi strain is in the case of typhoid.

Much more serological work needs to be done, and much work on the chemical nature of the antigen.

## *Summary*

A strain (L) of *Brucella abortus* is described, which possesses, besides the antigens A and M, a further antigen L. This antigen envelops the organism so as to render it inagglutinable by the usual rabbit anti-Brucella sera, though it is agglutinated by the sera of *Br. abortus* carriers. Heating to 100° C. for 30 minutes renders the organisms agglutinable by rabbit anti-Brucella sera, presumably by removing the L antigen from the bacterial surface.

Parallels with the Vi antigen of *S. typhi* are indicated, and it is suggested that the L strain might be of value in the detection of carriers of *Br. abortus.*

## **Addendum**

Further observations with strain L gave the results in table IV. Unfortunately strain L became rough after 9 months of intensive study. Lyophile dried samples are rough also. Criteria for roughness are: agglutination after 2 hours at 100 C. and spontaneous agglutination of the bacilli after treatment with acriflavine, 0·1 per cent.

TABLE IV

*Agglutination of different Brucella strains by sera obtained from rabbits and guinea pigs infected with strain L*

| Sera | Strains | | | |
|---|---|---|---|---|
| | 63 | L | M | S |
| K 20 | 800 | 1600 | 200 | 400 |
| K 20 absorbed with strain 63 | 0 | 400 | 0 | 0 |
| K 20 absorbed with strain L | 0 | 0 | 0 | 0 |
| K 45 | 400 | 400 | 100 | 200 |
| K 45 absorbed with strain 63 | 0 | 200 | 0 | 0 |
| K 45 absorbed with strain L | 0 | 0 | 0 | 0 |
| C zg | 200 | 200 | 0 | 0 |
| C bw | 100 | 200 | 0 | 0 |

63 = *Br. abortus* Bang. M = *Br. melitensis.*
L = the investigated Brucella strain. S = *Br. abortus-suis.*
K 20 and K 45 are sera of rabbits previously infected with strain L.
C zg and C bw are sera of guinea-pigs treated with the same strain. The rabbits were seriously ill and lost much weight. The guinea-pigs were ill also.

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# FURTHER OBSERVATIONS ON THE GRAM-TWORT STAIN

W. S. Ollett

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Since the publication of a method for staining Gram-positive and Gram-negative bacteria in sections (Ollett, 1947), we have had many enquiries about the method of preparing Twort's stain. This neutral red-light green mixture was prepared by Twort (1924) by mixing half-saturated aqueous solutions of the dyes and dissolving the precipitate so formed in 80 per cent. alcohol; the resulting stock solution was diluted with an equal volume of water to make the staining solution. Until recently we have used this method, substituting fast green FCF for light green to avoid fading.

We have recently found that a satisfactory stock solution can be made by mixing absolute alcoholic solutions of neutral red and fast green FCF. No *precipitate is formed, and this stock solution diluted 1 in 4 with distilled water* gives excellent results, identical with those obtained with the original Twort stain. The *p*H of this stain, like that of Twort's stain, is 4·9; this may depend on the dye samples we used, but uniformity in this respect could be secured by diluting the stock solution in *M*/5 acetate buffer instead of distilled water.

The proportions of the dyes given below are only approximate, and the optimum formula will depend on the dye content of the samples used. The stock alcoholic solution should be of a reddish magenta tint: too much green will weaken the red bacterial staining. The total dye concentration is less critical.

### *Modified Twort's stain*

Stock alcoholic solution

| | |
|---|---|
| 0·2 per cent. alcoholic neutral red (C. I. no. 825) . . | 90 ml. |
| 0·2 per cent. alcoholic fast green FCF . . . . | 10 ml. |

For use, dilute one volume of stock solution with three volumes of distilled water. Use as counterstain to Gram's method, as previously described (Ollett), but clear in monochlorbenzene instead of xylene and mount in L.P.M. The use of xylene leads to fading of the Gram's stain.

### *L.P.M. Mountant*

Lustron (L 2020) crystal clear 100 g.
Dissolve in a mixture of

| | |
|---|---|
| Dibutyl phthalate . . . . | 50 ml. |
| Monochlorbenzene . . . . | 300 ml. |

Lustron is apparently identical with "Distrene 80", which is now unobtainable.

I wish to thank Dr C. L. Oakley and Dr D. J. Trevan for their guidance and advice.

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576 . 851 . 49 (Salmonella) : 576 . 809 . 542

# MULTIPLICATION OF CERTAIN SALMONELLA SPECIES IN AUTOCLAVED BUFFERED TAP WATER

P. B. Crone

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In 1937, Bigger demonstrated that various strains of non-pathogenic coliform organisms were able to multiply in natural waters. He found that these waters, after being autoclaved, filtered or treated with various substances, would support growth more readily. It has been possible to extend his findings to include multiplication in buffered tap water of three species of Salmonella.

## *Experimental method*

To remove traces of organic matter all glassware was steamed for 6 hours in a chromic acid mixture (chromium trioxide 100 g., 20 per cent. (v/v) sulphuric acid to 1 litre). It was rinsed in several changes of tap water and finally in distilled water. Water to be tested was made into *M*/100 potassium dihydrogen phosphate-caustic soda buffer of *p*H 7 and 1 ml. quantities were put into $3 \times \frac{1}{2}''$ test tubes. These were plugged with cotton-wool and autoclaved at 10 lb. for 15 minutes. In this way tubes of autoclaved buffered tap water were prepared with Newcastle water, which, originating as an upland surface water, receives considerable treatment, including aluminium-sulphate precipitation, filtration and chlorination. The same was done with Sunderland water, chiefly a deep-well water, but containing a variable amount of unfiltered upland surface water. Tubes of autoclaved buffered distilled water were also made and used in exactly the same manner to serve as controls. The distilled water was produced in an all-glass still operated slowly. Slow distillation was essential, because rapid distillation caused splashing and the distillate supported growth.

With two exceptions, the organisms used were recently isolated from cases of food poisoning or enteric fever. They were taken either from platings of fæces on desoxycholate-citrate agar or from the same medium after enrichment in selenite broth. The two exceptions were from egg-slope cultures of organisms similarly isolated a year before. With a wire, tubes of the buffered water were inoculated direct from the plates. After incubation of the tubes at 37° C. for about 24 hours, a large loopful—about 0·01 ml.—was spread on a solid medium which was similarly incubated. If more than ten colonies appeared on the solid medium a subculture of an equal inoculum into another water tube was made. If less than ten colonies appeared, incubation of the tube was continued until more than that number were present. When, even after many days' incubation, no colonies developed from a loopful, the last tube to have a satisfactory count was used to start another series of cultures. In some cases, especially early in each series, subculture was carried out, without a count being made, on solid medium, each tube of the series being incubated for 24 hours. By the 9th subculture in water it could be confidently assumed that the organisms were growing on some substance in the water and not on nutrient carried over from the original plate.

## *Results*

Three strains of *Salm. typhimurium* and one each of *Salm. paratyphi* B and *Salm. enteritidis* were cultured in series in at least 10 tubes of Newcastle autoclaved buffered tap water. In addition each of these was passed through three transfers in Sunderland autoclaved buffered tap water at a late stage of the Newcastle series. One strain of *Salm. typhimurium* was passed through 10 cultures in Sunderland autoclaved buffered tap water direct from desoxycholate-citrate medium. Attempts to make similar series of cultures of 5 different strains of *Salm. typhi*, including one laboratory strain, were all unsuccessful. The third culture in autoclaved buffered tap water might have countable cells, but multiplication did not occur and the series beyond this failed.

The autoclaved buffered distilled water did not allow of repeated subculture with any of the strains mentioned. The organisms survived but did not multiply. Presumably lack of nutrient properties and not an inimical agent was responsible, for the addition of autoclaved buffered tap water to a suspension in autoclaved buffered distilled water led to multiplication.

In a single test, a tube of autoclaved buffered tap water sown with *Salm. paratyphi* B was incubated at 20° instead of 37° C. The organisms multiplied until the final count was 5 times the inoculum.

Final counts were variable with the three species that grew, but usually about $10^4$ organisms per ml. were present in the Newcastle autoclaved buffered tap water and about twice as many in Sunderland autoclaved buffered tap water. The rate of multiplication was low. It ranged from 20 times to less than 2 times in 24 hours.

## *Discussion*

Multiplication of organisms requires water, a source of energy and the elements carbon, nitrogen and others, alone or combined in varying degrees of complexity. In certain cases energy can be obtained direct from sunlight or from certain inorganic substances, but for all pathogenic organisms it is obtained, so far as is known, only from organic compounds. The minute quantities of these substances required to produce $10^4$ cells, of wet weight less than $10^{-7}$ g., might well be found in one ml. of tap water. Thus 1 per cent. peptone can be made to yield more than $10^9$ cells per ml., so $10^{-7}$ g. per ml. of utilisable organic matter would suffice for the production of $10^4$ cells per ml. Newcastle tap water is faintly brown, a colour no doubt derived from algæ, and such algal remains may be the nutrient used by the organisms studied.

It cannot be denied that the cotton-wool plugs of the tubes might provide nutrients. These could not alone be adequate, for they were unable to make autoclaved buffered distilled water a nutrient medium for the salmonellæ.

*Salm. typhi* is, in general, a more demanding organism, and its special need of tryptophane need not be the cause of its failure to grow in autoclaved buffered tap water.

There is no question, of course, of multiplication in untreated tap water. That from Newcastle contains sufficient chlorine to be rapidly lethal to salmonellæ; but, even in the absence of chlorine, the organisms naturally present, or some other cause, may prevent multiplication of a pathogen.

## *Summary*

On fresh isolation from cases of enteric fever and food poisoning, *Salm. paratyphi* B, *Salm. typhimurium* and *Salm. enteritidis* could be repeatedly subcultured in autoclaved buffered tap water. It was found impossible to maintain *Salm. typhi* in series by these means.

I am indebted to Dr G. S. Wilson for advice and criticism.

## REFERENCE .

BIGGER, J. W. . . . . . . . 1937. This *Journal*, xliv. 167.

# BOOKS RECEIVED

## The actinomycetes

By SELMAN A WAKSMAN 1950 Waltham, Mass The Chronica Botanica Co London William Dawson and Sons, Ltd Pp xviii and 230, 64 text figs 30s

In spite of the great interest taken in the actinomycetes during the last half century, remarkably little has been written on the more general aspects of the subject Lieske, in his monograph published in 1921, did much to collate the then available information, but the subject has grown apace since that time and the need for a comprehensive and up to date review has been felt for many years No one is better qualified than Professor Waksman to undertake this task and there is consequently little doubt that *The actinomycetes* will be regarded as a notable contribution to the literature

One of the great difficulties that has faced students in the past has been that of assessing the value of much of the literature because of the confused terminology and the multiplicity of generic names used by the earlier workers These perplexing problems of classification, taxonomy and morphology have been dealt with in admirable fashion by the author, whose experience has enabled him to present a useful summary and discussion of all the systems of classification to date, together with an exposition of his own system—propounded jointly with Henrici—which is almost certainly the simplest and most easily workable yet devised The chapters on variation, distribution and physiology are no less important and informative

In the main, research workers have devoted their attention to three important fields, the nature of the causative organisms of human and animal actinomycoses, the cause, nature and control of potato scab and, in more recent times, the isolation and production from actinomycetes of antibiotic substances, several of which are of sufficient importance to have a profound effect on clinical practice Workers in these fields and particularly in the last mentioned, which offers many opportunities for useful work, will find much of interest and value to them in this volume

In *The actinomycetes*, Professor Waksman has succeeded in bringing together most of the existing information and presenting it in a concise and readable form Primarily, this is a work of reference made especially useful by its extensive bibliography, and there is no doubt that it will commend itself to the general microbiologist and the specialist alike

## Toxæmias of pregnancy

Edited by JOHN HAMMOND, F J BROWNE and G E W. WOLSTENHOLME 1950 London J & A Churchill, Ltd Pp xii and 280, 15 plates (29 figs) and 65 text figs 21s

This volume is composed of 29 papers which were presented at a symposium on toxæmias of pregnancy held in London in January 1950, The contributors are drawn from the United States of America, the British

Commonwealth and Europe, as well as from all parts of Great Britain, and the subject is dealt with from the point of view of both human and veterinary medicine.

Pathologists will regret the brevity of Sheehan's contribution on the pathological lesions of eclampsia and pre-eclampsia. It is unfortunate that time and space were not available for a more detailed presentation of the evidence upon which Sheehan's views are based, for many of his descriptions differ materially from the orthodox accounts. The experimental studies of Dieckmann and his assistants on the effects of sodium chloride and lactate injections and the careful studies of the hæmodynamics of the circulation in normal and toxæmic pregnancy presented by Kellar, Hamilton and Burt are important original contributions to the pathogenesis of this disease. Schneider's work on the effect of thromboplastin injections is interesting but its applicability to the problem under discussion seems doubtful. Bastiaanse and Mastboom elaborate a hypothesis that toxæmias result from ischæmia of the gravid uterus but they do not cite any evidence that ischæmia does in fact occur. Glynn summarises recent work on the association of hepatic lesions with dietetic deficiencies. Somerville's account of the relationship between the placental hormones and the toxæmias stresses the difficulties of any interpretation of the findings in this field ; there is clearly room for more detailed studies in this direction. Thomson on the mono-amine oxidase activity of the placenta and Ahlmark, Werkö and Kapeller-Adler on histaminase activity present interesting accounts of enzyme action which may be disturbed in toxæmic pregnancy. Toxæmias of pregnancy occurring in sheep, cattle and rats are discussed in a group of 6 papers. It seems clear that most of these conditions bear little relationship to the human diseases.

The general effect of this book is disappointing. The large number of short independent communications has led to a lack of integration, so that while many interesting details of the problem of the toxæmias are discussed, there is nowhere a synthesis of the various aspects into a general view of the subject as a whole.

## Notes on communicable diseases of laboratory animals

By H. J. Parish. 1950. Edinburgh : E. & S. Livingstone, Ltd. Pp. vii and 69. 3s.

*Multum in parvo* aptly describes this book of notes on the communicable diseases of laboratory animals. In the space of 69 pages are mentioned no less than a hundred types of infection in eleven animal species. Each species is considered in turn, the general plan being to note whether the disease is a common one ; whether or not it is transmissible to man or to other animals ; the symptoms ; the causal organism (where known) and the most useful methods of control. The information presented is based on sound bacteriological principles combined with reasonable common-sense and it is obvious that the author speaks from personal experience. It would have been useful to have had a little more information about the fairly commonly observed " rolling or twisting " in mice, although there is a good description of the condition in rats. Infectious renal cortical necrosis in the mouse is not mentioned and the section on the cat is rather scanty. The statement (p. 20) that " Injudicious feeding, etc., may light up infection " in rabbits is not very helpful. But this is not a handbook of husbandry and within their own limitations these notes should be helpful in furnishing the clue to many of the sporadic diseases and epizootics which from time to time are the despair of all workers with laboratory animals.

## Types of diabetes mellitus and their treatment

By Arthur R. Colwell. 1950. Springfield, Ill.; Charles C. Thomas: Oxford; Blackwell Scientific Publications, Ltd. Pp. v and 97; 6 text figs. 16s. 6d.

This small monograph covers most aspects of diabetes from diagnosis to treatment of coma. It is well produced and clearly written by an author of high reputation with many worth-while publications on his subject. But it is difficult to know what useful purposes this book serves. It does not contain sufficient detail to enable a practitioner to undertake treatment from its pages or to interest an expert by its theoretical considerations. In some directions the author holds conservative views, especially when he advocates a C-Fat ratio in the diet of 1 to 1 as most generally useful and " the optimal ratio with insulin ".

The title of the book is stimulating and led the reviewer to hope that various different fundamental types were to be described. It turns out, however, that it is not different types that are referred to, but grades of diabetes—mild, moderate and severe. The title therefore merely makes an already well-known quantitative distinction and not new qualitative ones.

## Practical section cutting and staining

By E. C. Clayden. 2nd ed., 1951. London: J. & A. Churchill, Ltd. Pp. vii and 135; 8 figs. on 4 plates and 15 text figs. 10s.

This second edition of Mr Clayden's little book is for all practical purposes a copy of the first edition (see this *Journal*, 1948, lx, 527). There have been added two or three additional methods dealing with the preparation of bone sections and the staining of fat and myelin. A few further additions might have been made with advantage, such as the Scharlach-R method for fat, the Dobell modification of Levaditi's method for spirochætes and the phosphotungstic acid-hæmatoxylin method of Mallory (P.T.A.H.) for fibrin and neuroglia. Mention might also have been made of the use of the ultra-violet lamp in von Kossa's silver method for calcium.

The book retains its original form as a handy work of reference for the routine laboratory bench and will doubtless have a reception not inferior to that of the first edition. It is a very useful book.

## Pathologische Histologie

By Max Borst. 4th ed., revised and enlarged, 1949. Munich: J. F. Bergmann. Pp. xii and 539; 405 text figs. (199 in colour). DM 94·50 (unbound).

The publication of this edition has been delayed for nearly four years after the death of its distinguished author. The only notable addition to the 3rd edition (1938) is a new chapter devoted to tumours of the central and peripheral nervous system within the space of 30 pages inclusive of 34 drawings. The classification closely follows that of Bailey and Cushing with the consequent threat of bewildering the medical student and the doctor to whom it is addressed. In the upshot, this chapter, better than any other, exemplifies the author's skill in the lucid exposition of a large and complex subject with a moderate number of drawings and remarkably few words.

To the end, in successive editions of his book, Borst adhered to certain views which most of his contemporaries agreed to abandon. Thus he derives the hypernephroma of the kidney from adrenal rests although he admits (p. 477) that such tumours occur "nur äusserst selten" in the adrenals themselves; he attributes the symptom complex of Addison's disease to a lesion of the adrenal medulla rather than of the cortex (p. 362), and Simmonds's disease (p. 364) to inflammatory lesions of the anterior lobe of the pituitary. Despite these and other idiosyncrasies of opinion, however, this edition, like its predecessors, should be of great interest to teachers of pathology in this country. How should pathology be taught to the medical student—and how much of it? As Borst explains, the first edition was published in 1921 in fulfilment of a wish repeatedly expressed by his students over many years. It embodied an educational course which had proved appropriate to its purpose ("zweckentsprechend") in 25 years' experience of teaching and which was presumably maintained unchanged during the next 25 years. It is more than a manual of pathological histology because, wherever possible, it seeks to correlate pathogenesis and symptomatology with histological pattern. It throws what we are pleased to call "general principles" to the four winds and immediately tackles the description of special pathology, organ by organ, including 10 pages on the skin. In this way, Borst avers, "general pathological histology comes into its own", which is interpreted as meaning that general principles will look after themselves. Anyhow his book scored an immediate and lasting success with the German student body which is probably not less philosophical, on the whole, than the British.

# PROCEEDINGS OF THE PATHOLOGICAL SOCIETY OF GREAT BRITAIN AND IRELAND

*29th and 30th December* 1950

The eighty-second meeting of the Society was held in two sections in the Department of Pathology, St Thomas's Hospital Medical School, London, on Friday and Saturday, 29th and 30th, December 1950.

### Communications and demonstrations

Items marked with an asterisk are abstracted below

R. E. Rewell. Testicular tumours of birds.

B. Lennox and A. L. Wells. Differentiation in the rodent ulcer group of tumours.

G. J. Griffiths, A. P. Hayhurst and Raymond Whitehead. Dissecting aneurysms of the aorta in mother and child.

R. E. Billingham, J. W. Orr and D. L. Woodhouse. Epidermal transplantation during chemical carcinogenesis: the effects of reciprocal transference between carcinogen-treated and untreated skin sites.

K. C. Dixon and B. M. Herbertson. Lipids of normal and tabetic spinal cord.

*K. C. Dixon and B. M. Herbertson. Cytoplasmic glycolipids of brain.

J. A. Milne and J. B. Rennie. The effect of 3-hydroxy-2 phenyl-cinchoninic acid on generalised scleroderma.

H. E. Hutchison. The effect of adrenalin on the absorption of tetanus toxin from granulating wounds prepared by the " viskap " method.

R. J. V. Pulvertaft. Phagocytosis.

J. H. Humphrey. The effect of cortisone on the Arthus phenomenon.

L. E. Glynn and G. Loewi. The nature of fibrinoid necrosis.

J. V. Dacie and C. de Gruchy. The demonstration of hæmolytic antibodies in acquired hæmolytic anæmia.

*A. Glucksmann. The histogenesis of radiation-induced and of benzpyrene-induced epidermal tumours in the mouse.

G. F. M. Hall. The significance of atheroma of the renal arteries in diabetic glomerulosclerosis.

P. T. J. C. P. Warner, J. V. T. Gostling and A. C. Thackray. The effect of freezing and freeze-drying on sarcoma 37.

J. G. Carr and R. J. C. Harris. The preservation of the virus of the Rous fowl sarcoma.

*J. Craigie, P. E. Lind, M. E. Hayward and A. M. Begg. Preliminary observations on a " dormant " state of sarcoma cells with special reference to resistance to freezing and drying.

E. M. Darmady and Daphne R. Jones. The study of the nephron in acute tubular necrosis by the Oliver technique.

E. Topley and E. Lowbury. The value of polymyxin in burns.

W. M. Davidson, Sylvia D. Lawler and A. G. Ackerley. The Pelger-Huet leucocyte anomaly.

N. P. Markham and J. H. Kent. A technique for giving repeated intravenous injections to guinea-pigs.

B. M. Wright. A comparison between the histological reaction to coal-dust in human and animal lungs.

R. I. K. Elliott, H. Kramer and A. H. T. Robb-Smith. A comparison of wound healing by granulation in scorbutic- and cortisone-treated guinea-pigs.

C. E. Lumsden. Normal oligodendrocytes in tissue culture.

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J. Ungar. The effect of varied amounts of aluminium salts on pertussis and diphtheria antigens.

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G. B. Ludlam. Pharyngeal inclusion bodies in new-born infants.

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C. G. Pope. Observations on the purity of diphtheria toxin.

C. N. Iland. Some observations on the serology of polysaccharide fractions of *M. tuberculosis.*

C. N. Iland, G. S. Gilbert, A. S. Jones and R. W. Baldwin. A new antigenic fraction from *M. tuberculosis.*

*R. R. A. Coombs, L. S. Mynors and F. Wild. *The recognition and titration of antibodies to chemical substances by the agglutination reaction.*

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**Abstracts**

576.311.1:611.81

## CYTOPLASMIC GLYCOLIPIDS OF BRAIN

K. C. Dixon and B. M. Herbertson

*Department of Pathology, University of Cambridge*

Two glycolipids are commonly present in the cytoplasm of neurones of the central nervous system. Both are stained by periodic acid and leucofuchsin as well as by sudan black. The first of these is diffusely distributed in the cytoplasm, being in close proximity to the Nissl substance (Dixon and Herbertson, 1950*a*). It can be extracted by a hot ethanol-ether mixture which does not remove the pyronin staining of the Nissl substance (due to nucleic acid). This diffuse glycolipid is present in high concentration in the chromophilic cells of the cerebral cortex of the rabbit and rat and in many of the Purkinje cells of the cerebellum of these animals.

The second glycolipid has so far only been observed in human neurones (Dixon and Herbertson, 1950*b*); it is pigmented to a variable degree, and is in fact either associated or identical with the well-known lipofuscin or wear-and-tear pigment. It occurs as condensed clusters of granules within the cytoplasm.

We now find that the diffuse glycolipid is probably also present in many human neurones, although generally in much smaller amounts than in the Purkinje cells of the cerebellum and the chromophilic cells of the cerebral cortex of the rabbit and rat. In human material it is readily seen in thick (17 $\mu$) sections of medulla and spinal cord, where greater depth of the large cell bodies can be stained than in the usual routine (5 $\mu$) sections. In thin sections of human material the diffuse glycolipid is faintly stained by periodic acid and leucofuchsin. We are grateful to Dr J. B. R. Cosgrove for showing us a section of sheep medulla which he had stained with periodic acid and Schiff's reagent. In this section the localisation of the diffuse glycolipid in the Nissl granules was more prominent than in our material, although we had previously observed (Dixon and Herbertson, 1950*a*) the close association of the diffuse glycolipid of rabbit neurones with the Nissl substance. In human material this association is also evident. We have succeeded in extracting much of the diffuse material reacting to periodic acid and leucofuchsin from the neurones of human medulla by re-fluxing with boiling chloroform. Such treatment does not substantially diminish the staining of the lipofuscin granules.

We suggest that in the normal metabolism of the neurone the pentose nucleic acid of the Nissl granule is architecturally incorporated in a dispersed complex with the glycolipid which reacts with periodic acid and leucofuchsin. It is known that pentose nucleic acid is destroyed in chromatolysis produced by excessive activity, trauma or infection. It appears possible that continued incidents of this kind, occurring over many years in the history of the neurone, might result in the glycolipid being expelled from the dispersed complex when the pentose nucleoprotein, with which it is associated, is destroyed. In this way aggregates of glycolipid are formed as clusters of granules which are prominent in the neurones of the aged. Such inclusions outside the sphere of cytoplasmic metabolism might then become permanent landmarks of past neuronal events.

### REFERENCES

Dixon, K. C., and Herbertson, B. M., 1950*a*. *J. Physiol.*, cxi, 244.

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After about 2 months warts appear and after 3 months malignant epitheliomas arise in either the hyperplastic regions or the warts (Glucksmann, 1945).

The carcinogenic hydrocarbons thus lead rapidly to tumour formation by direct stimulation of the treated epidermal cells, while radiation produces tumours slowly and indirectly in the regenerating tissue after the destruction of the directly irradiated epidermis and derma and after causing severe vascular damage.

The initial stimulating effects of benzpyrene produce a reversible hyperplasia and reversible warts; they produce potential cancer cells which are rendered irreversibly malignant by further stimulation. The initial destructive effects of irradiation are irreversible, but indirectly they too stimulate the growth of the adjacent regenerating tissue. The response of this tissue diminishes progressively and becomes irregular—as it also does in the benzpyrene painted skin—and results in malignancy. In both cases the epidermis appears to be unable to respond to growth stimulation beyond a certain degree and yet to differentiate normally. This inability to combine rapid or prolonged proliferation with normal differentiation seems to be one of the characteristics of carcinogenesis. The concept of carcinogenesis due to an adaptive variation of a tissue rather than to a somatic mutation and subsequent proliferation of individual cells seems to fit our experimental findings.

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616—006.42—018.21:578.083

# PRELIMINARY OBSERVATIONS ON A "DORMANT" STATE OF SARCOMA CELLS WITH SPECIAL REFERENCE TO RESISTANCE TO FREEZING AND DRYING

JAMES CRAIGIE, P. E. LIND, M. E. HAYWARD and A. M. BEGG

*From the Laboratories of the Imperial Cancer Research Fund, London*

The effects of isotonic dextrose solution on tumour-cell suspensions are paradoxical. Exposure of a cell to dextrose solution produces a profound and almost instantaneous change in the nucleus. The nucleoli and associated chromatin, clearly visible under phase-contrast illumination, become invisible and the nucleus swells. This change, together with the progressive disintegration of cytoplasm which follows, would appear to be incompatible with cell survival. On the other hand, it is well established that tumour-cell suspensions preserved in the frozen state in dextrose will produce tumours when thawed rapidly and injected without delay (Craigie, 1949*a*).

Quantitative studies of sarcoma-cell survival in fluid suspension *in vitro*, carried out on lines previously indicated (Craigie, 1949*b*) have yielded similar paradoxical results. Suspensions of C3H sarcoma cells were prepared in various isotonic fluids (extracellular and intracellular physiological fluids, etc.) and samples were examined and tested at intervals from 24 hours to 21 days. The volume of surviving viable cells was estimated from the mean time of tumour appearance following subcutaneous inoculation of mice, and in order to determine the correlation between details of cell morphology and viability, photomicrographic (phase-contrast) records were made of samples of suspension mounted on cytometer slides. It should be noted that in work of this kind it is essential to avoid fallacious results due to inadequate or non-uniform exposure of cells to the suspending fluid. Clumps of cells must be eliminated and the ratio of cell volume to suspending fluid should not exceed 1 to 50.

The results obtained may be briefly summarised by stating that under the conditions employed there is no correlation between the number or apparent state of preservation of " normal " C3H sarcoma cells and the volume of viable cells estimated by titration in mice. Since the tumour-inducing activity of treated sarcoma-cell suspensions cannot be attributed to survival of obviously damaged " normal " tumour cells, some other explanation is required. Recent observations indicate that dense refractile cells found in some tumours are viable and possess the resistant properties necessary to explain the paradoxical results obtained in studies of tumour-cell survival.

Refractile tumour cells are easily distinguished from " normal " tumour cells by phase-contrast microscopy. Their high refractive index obscures the details of their internal structure and produces a brilliant and characteristic image. Round to spindle forms occur, according to the cell type of the tumour. These cells are known to occur in the following transplantable mouse tumours :—C3H sarcoma, sarcoma 37, C 57 sarcoma and carcinoma 63, all of which can be preserved in the frozen state as suspensions in dextrose.

When suspensions of refractile $C_3H$ sarcoma cells are fixed, cut and stained, the cytoplasm is seen to be condensed and acidophilic and the nucleus dense. In this respect they resemble cells which might be identified in tumour sections, either scattered in small groups without any obvious concomitant pathological change or, with less certainty, bordering necrotic foci and scattered among the other cells, necrotic debris and associated fluid.

The percentage of cells found in the refractile state in suspensions freshly prepared from subcutaneous tumours is unpredictable and very variable, but averages about 5 per cent. in sarcomas 37 and C3H. Quantities of refractile cells in a relatively pure state may be obtained by fractionation of the ascitic fluid associated with C3H sarcoma or, better, sarcoma 37 in the C3H strain of mice.

*In vitro*, refractile tumour cells are relatively stable when freshly isolated, but later they undergo striking changes which would seem to indicate that they are viable. The first stage in in-vitro transformation is swelling of the cell. As this progresses refractility decreases and the nucleus gradually becomes visible. At the same time the cytoplasm extends irregularly into processes of extremely variable shape. Potocytosis develops, then ceases, and is followed by nuclear and cytoplasmic changes indicative of death of the cell.

The results of subcutaneous mouse inoculation confirm the view that refractile cells are viable. Eight hundred C3H refractile cells will produce a visible tumour (3 mm.$^3$) in 8 days. Most refractile cells survive freezing and thawing in dextrose solution and some survive drying from the frozen state. For example, after drying in a single-cell layer in dextrose at $-20°$ C., 0·2 per cent. of an almost pure suspension of S 37 (ascitic) refractile cells remained in the refractile state. Eight mice received an average subcutaneous dose of 75 of these surviving cells. Five mice showed tumours 12 days after inoculation and seven were found to have developed tumours by the 21st day.

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# THE RECOGNITION AND TITRATION OF ANTIBODIES TO CHEMICAL SUBSTANCES BY THE AGGLUTINATION REACTION

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Landsteiner and Lampl (1917, 1918) showed that antibodies could be produced against groups of known chemical constitution, such as atoxyl, by inoculating rabbits with the compound coupled with a foreign protein by the azo linkage. Since that date much work has been done by Landsteiner and his school and others with such artificially conjugated antigens and on serological reactions with simple chemical compounds.

Antibodies produced in this way against a chemical substance are usually demonstrated by means of the precipitation or complement-fixation reaction. In these in-vitro tests the chemical substance is diazotised and coupled to a protein immunologically distinct from that used for the inoculations. In addition, the resulting precipitation may be specifically inhibited by a solution of the chemical itself.

In a previous paper Coombs, Mynors and Weber (1950) described a method for the recognition and titration of antibodies against atoxyl by means of an agglutination reaction. In the detection of the antibody this technique was more sensitive than the precipitation reaction. The present work is a continuation of these studies.

The two compounds studied in the present investigation were atoxyl (arsanilic acid) and sulphanilic acid. Antisera which would react specifically with these determinant groups were produced by inoculating rabbits with each compound coupled with egg albumin by the azo linkage. For brevity and simplicity these two antigens are referred to as egg albumin-atoxyl and egg albumin-sulphanilic acid respectively.

To obtain a stable yet agglutinable red-cell suspension possessing the specificity of the determinant group under study the following method was used. Before the performance of the test the proteins of a human serum containing an " incomplete " or non-agglutinating form of Rh antibody (anti-D) were conjugated with the determinant group in question. In the test itself Rh-positive human red cells were incubated at 37° C. with the treated serum in order to absorb the conjugated incomplete antibody. The cells, after a thorough washing in saline, formed a stable suspension which could be agglutinated specifically by antisera prepared against atoxyl or sulphanilic acid, as well as by antisera prepared against human serum globulin.

The coupling of up to 15 groups per Rh antibody molecule did not destroy the specific combining power of the antibody, although with certain sera the demonstrable antibody titre was considerably reduced.

The table shows the results of titrating the agglutinating property of the anti-atoxyl and anti-sulphanilic acid sera against cell suspensions carrying the respective determinant groups. The results clearly illustrate the specificity of the reactions. Before testing, all the sera were absorbed free of agglutinins for normal human red cells. A further criterion of the specificity of these reactions was demonstrated by specific-inhibition tests using a 0·25 per cent. solution of either atoxyl or the sodium salt of sulphanilic acid ; only the homologous reactions were inhibited.

The experiments are still in a preliminary stage, nevertheless the method promises to have general application to those aspects of immuno-chemistry concerned with artificially conjugated antigens. The primary object of this and subsequent work is a study of the drug allergies. The intention is to see whether by an adaptation of this technique it is possible to demonstrate in the sera of such cases a circulating specific reagent.

TABLE

*The agglutinating titres of anti-atoxyl and anti-sulphanilic acid sera against cell suspensions carrying the respective determinant groups*

| Titration of sera | Against | | | |
|---|---|---|---|---|
| | Untreated human red cells | Human cells sensitised with untreated incomplete *D Rh antibody* | Human cells sensitised with incomplete D Rh antibody conjugated with diazotised atoxyl | Human cells sensitised with incomplete D Rh antibody conjugated with "diazotised" sulphanilic acid |
| Pre-inoculation rabbit sera | 0 | 0 | 0 | 0 |
| Rabbit anti-egg albumin . | 0 | 0 | 0 | 0 |
| Rabbit anti-egg albumin-atoxyl | 0 | 0 | 160 | 0 |
| Rabbit anti-egg albumin-sulphanilic acid | 0 | 0 | 0 | 160 |
| Rabbit anti-human globulin | 0 | >640 | >640 | >640 |

0 = <2

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This number is dedicated to
Professor Carl H. Browning, F.R.S.,
on the occasion of his 70th birthday,
21st May 1951

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# CARDIO-VASCULAR RESPONSES TO AIR EMBOLISM

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While working on pulmonary œdema (Cameron and De, 1949) we became interested in air embolism, and we noted that, from time to time, animals showed curious variations in their susceptibility to this condition. We now report some experiments we have performed for the purpose of analysing cardio-vascular responses to pulmonary embolism, from which we conclude that the most important factor in fatal cases is blockage of the pulmonary circulation, although myocardial disturbance may play a part.

## Methods

Our observations have been made on fully-grown, well-nourished male rabbits, 1·9-3 kg. body weight, anæsthetised with intravenous Nembutal in doses of 40 mg./kg. body weight, followed by a little ether when vessels were cannulated for blood-pressure recordings and when electrodes were inserted subcutaneously. Most of our experiments dealt with air embolism, ordinary atmospheric air being introduced through a left ear vein by displacement with water under 200 mm. Hg. pressure at varying rates. The device for this is shown in fig. 1, where C denotes a screw clip for manipulation of the rate of injection. Embolism was also produced by intravenous injection of an emulsion of potato starch prepared by Dunn's method (1919-20), of a finely emulsified mixture of olive oil and lecithin and by the direct introduction of rape seeds. In this we made use of a slightly curved glass cannula of 2 mm. internal diameter, capable of holding about 100 seeds. It was connected by rubber tubing to the nozzle of a 20 c.c. glass syringe filled with normal saline at body temperature. The cannula could be introduced into an exposed jugular vein and the seeds run into the venous circulation along with a small quantity of saline.

The right-auricular pressure was recorded throughout the experiments by means of a plastic catheter inserted into the right auricle by way of the right external jugular vein and connected with a saline manometer and kymograph.

The carotid pressure and respiratory movements were simultaneously recorded with a mercury manometer and a cuff wrapped around the chest respectively, both recording on a kymograph. E.C.G. tracings were obtained by placing spatulate German silver electrodes, 4·5 × 1·0 cm., in the subcutaneous tissues. The three standard limb leads were employed. In the preliminary experiments tracings were made with a string galvanometer of standard speed and sensitivity, but because of difficulty in interpretation a direct recording apparatus (Cossor model) was substituted which was run four times faster than the normal speed, namely 1 cm. per second, and standardised to give a value of 2 millivolts for each 2 cm. excursion. Under such conditions, the various components of the E.C.G. curve are adequately spread out and easily decipherable. No interference by muscle potentials was encountered.

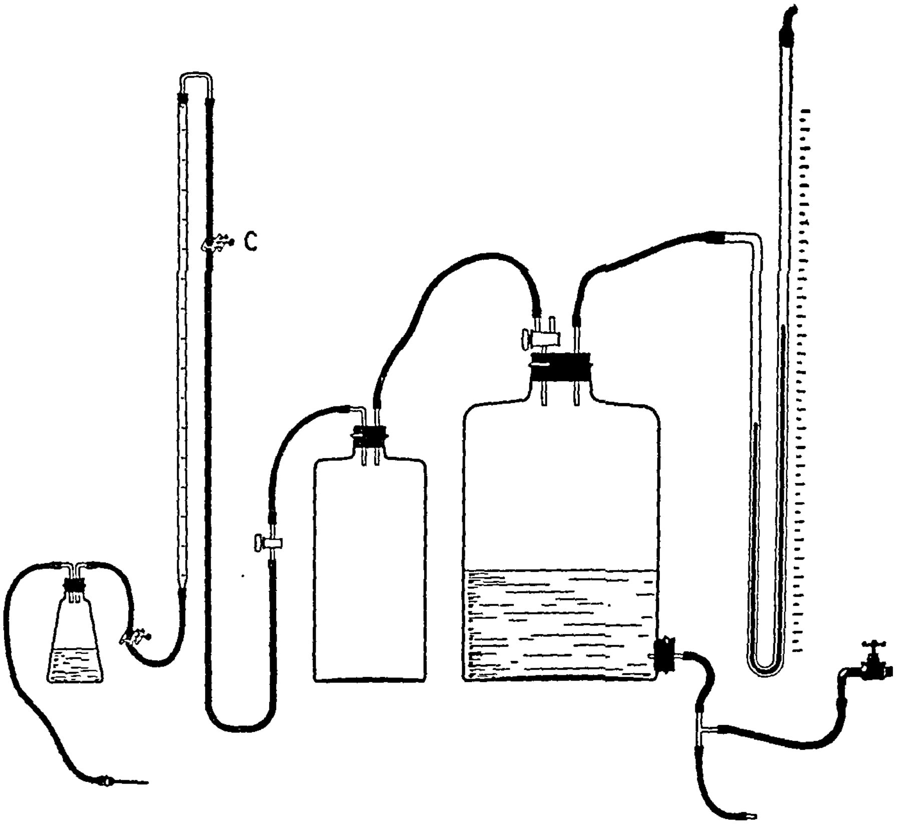

Fig. 1.—Diagram showing the arrangement for air injection by displacement with water under pressure. C is a screw clip for manipulation of the rate of injection.

At the conclusion of each experiment, a careful autopsy was performed and a close study of the heart, blood vessels and lungs was made under water.

## Results

### 1. *Blood pressure and respiratory changes produced by air embolism in the intact animal*

That a relation exists between the rate of injection of air and the time of onset of death has long been recognised and is well brought out in our experiments (table I). It will be seen that as the rate increases, the survival time decreases, although the total amount of air thus introduced is by no means constant. The carotid arterial pressure falls as the right-auricular pressure increases (fig. 2), the change in pressure being either gradual and spread over a fairly long time interval, or abrupt. Respiration rate is at first increased but becomes slow and irregular as embolism progresses.

TABLE I

*Representative experiments on air embolism in rabbits*

| Rate of injection of air (c c per min ) | Time of death (seconds) | Total amount of air injected (c c ) |
|---|---|---|
| 1 | 520 | 8 6 |
| 1 | 475 | 7 9 |
| 2 5 | 225 | 9 4 |
| 3 | 295 | 14 9 |
| 8 | 80 | 10 6 |
| 13 | 40 | 8 6 |
| 2 5 (bilateral vagotomy) | 270 | 11 2 |

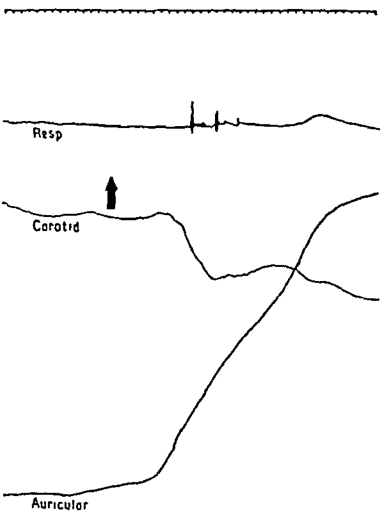

FIG 2.—Simultaneous recordings of respiration and of carotid and right auricular pressures before and after injection of 8 c c of air per minute intrajugularly. The arrow marks the commencement of air injection

If bilateral vagotomy be performed before the air is injected into the veins, respiration increases in depth and becomes slower. Such changes are exaggerated by embolism, but the carotid and auricular pressure variations are similar to those recorded in the intact animal and the lethal amount of air remains unchanged. These results suggest that a vagal reflex from the lungs does not give the circulatory disturbances associated with air embolism.

### 2. *The condition of the cardio-vascular system in air embolism*

The right ventricle and auricle, the great veins, the coronary veins and the pulmonary artery become distended with frothy blood, often to a remarkable extent. In contrast, the left auricle and ventricle, the pulmonary veins and the systemic arterial system are quite free from air bubbles and contain little blood. Despite careful search we have never found air bubbles in the coronary arteries or the left heart.

In a few experiments air was introduced intravenously with the animal under artificial respiration and the heart exposed through a reflected sternum. Bubbles were seen to enter the coronary veins some time before the right heart dilated and the carotid pressure began to fall. In other words, air bubbles appear within the coronary veins early during embolism before the heart action begins to fail.

### 3. *Electrocardiographic findings in air embolism*

Each of our 10 rabbits with air embolism showed definite alterations in their E.C.G. pattern. A deep S wave appeared in lead I, together with a prolonged downward deflection of the R.S.T. interval, in which the T wave was submerged. The S.T. interval often terminated in a short elevation, indicating an upward component of the T wave. A slight but definite depression of the S.T. segment was also observed in lead II. The T wave, however, assumed an inconstant position, either appearing submerged in the S.T. interval, flattened or upright. In lead III a large, upright T wave was invariably noted, although it was flattened in one animal. The S.T. segment remained near the zero line in lead III except in two cases where it experienced an upward deflection. A pronounced Q wave was encountered once only.

Other cardiac irregularities in these animals included frequent ventricular extrasystoles and sino-auricular and auriculo-ventricular block. In contrast to some other workers, we did not encounter ventricular fibrillation.

Sinus slowing was observed twice and partial heart block on three occasions. We noted that ventricular extrasystoles were more numerous soon after the introduction of air emboli and that sometimes short paroxysms of ventricular ectopic beats developed at this time.

The heart rate became slower in all but one of the animals; in this rabbit it was rapid at first but slowed down in the later stages. By the slow introduction of air bubbles and simultaneous recording of carotid pressures we could show that E.C.G. disturbances first appeared when the systemic blood pressure fell rapidly (figs. 3 and 4).

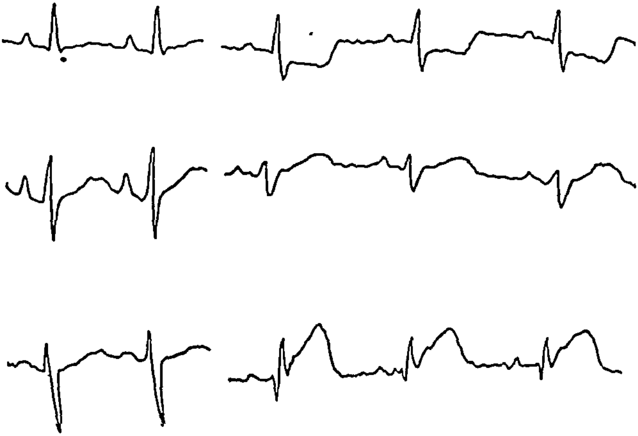

FIG. 3.—E.C.G. tracings before and after intrajugular injection of 5 c.c. of air at the rate of 1 c.c. per minute. Tracings taken when carotid blood pressure was 20 mm. Hg. and pulse rate 300 per minute. Left hand curves normal, right-hand curves due to air injection. Lead I top curves, lead II middle curves, lead III bottom curves. Lead I shows well-marked depression of S.T. segment with hurried T wave; rhythm regular. Lead II shows S.T. segment at the iso-electric line; T upright. Lead III: a Q wave is visible; S.T. take off is at a higher level than normal, T wave is large and upright.

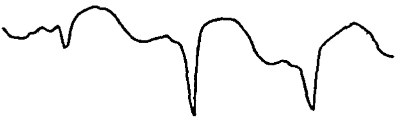

FIG. 4.—Lead III tracings after injection of 1 c.c. of air intrajugularly. Carotid blood pressure 60 mm. Hg., pulse rate 330 per minute. Paroxysms of ventricular extrasystoles.

Bilateral vagotomy did not affect these disturbances of pattern. One vagotomised animal developed a mild tachycardia with embolism, but the other rabbits showed the usual bradycardia. Since respiration is accelerated in the early stages of embolism, and might possibly influence the E.C.G. response, at the suggestion of Dr E. Pochin we induced rapid respiratory movements in normal rabbits by making them inhale carbon dioxide, but we were unable to detect any significant alteration in the E.C.G. pattern of such animals.

### 4. *Circulatory changes with other forms of pulmonary embolism*

Fat, starch and rape-seed embolism gave arterial and auricular pressure disturbances similar to those with air embolism (fig. 5). E.C.G. changes with fat and rape-seed embolism resembled those obtained with air embolism (fig. 6). Bilateral vagotomy did not affect the result. The interest of the rape-seed experiments is that no question of coronary-vein embolism arises, as with air embolism. The mean diameter of the rape seeds used was greater than that of the coronary veins we measured in rabbits and on no occasion did we find rape-seed emboli in structures other than the pulmonary arterial branches. Fat and starch emboli were located within the smallest pulmonary arteries and arterioles and sometimes as thin layers within capillaries. These experiments suggest that the main cause of the blood-pressure and E.C.G. disturbances is blocking of the pulmonary circulation. Interference with the outflow of blood from the coronary venous system, reflex vagal action on the heart and mechanical blockage of the coronary arteries were eliminated as factors. We would also emphasise that we obtained no evidence of

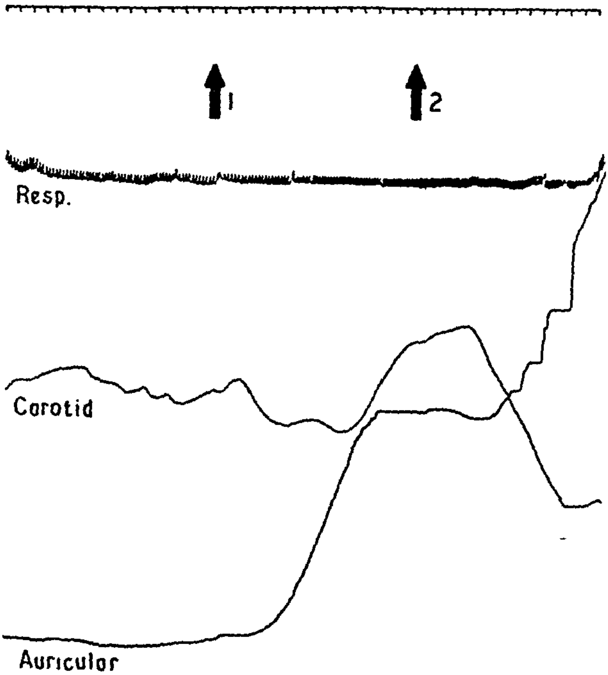

FIG. 5.—Simultaneous recordings of respiration and of carotid and right auricular pressures before and after seed embolism which was produced in 2 stages (arrows 1 and 2).

passage of any of these emboli through the pulmonary circulation to the brain, so that a direct cerebral factor may also be excluded.

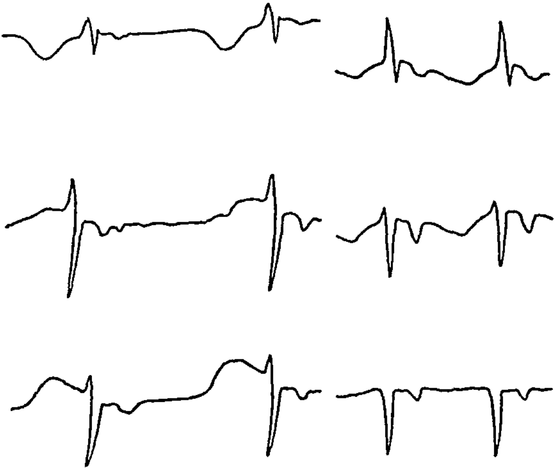

FIG. 6.—E C G tracings after intra jugular injection of about 50 rape seeds. B P. mm Hg Curves on left obtained before embolism, on right after embolism Lead I top curves, lead II middle curves, lead III bottom curves Lead I shows S T. segment greatly depressed, with T wave buried and with deflections of high amplitude Lead II shows a low, almost flat T wave and a low take off of S T Lead III shows a high upright T wave. P R and Q R S normal S is shorter than usual Notchings of unknown origin are seen in some complexes.

This, too, can be done with air embolism, a conclusion also reached as the result of careful experimental studies by Richardson *et al.* (1937), Curtillet (1939) and Kent and Blades (1941-42).

## DISCUSSION

Air embolism has interested pathologists for nearly three centuries and has been the subject of much experimentation. Morgagni (1769) says that Brunner and Verdriesius confirmed the experiment carried out by Wepfer, " who only by inflating with his mouth the jugular vein, did, once on a time, lay prostrate and kill an ox, of a stupendous size ". The date of the Wepferian experiment is not known, Morgagni finding no mention of it in any of Wepfer's writings published before 1667. However, Redi (1667) states that he had killed instantly by the same procedure 2 dogs and a hare, and " within the sixteenth part of an hour, in like manner ", a sheep and 2 foxes. A vivid description of the clinical features and *post-mortem findings* in a variety of animals is given by Heydius (1683, quoted by Morgagni), them inhale carbon dioxide, but we were unable to detect any significant alteration in the E.C.G. pattern of such animals.

### 4. *Circulatory changes with other forms of pulmonary embolism*

Fat, starch and rape-seed embolism gave arterial and auricular pressure disturbances similar to those with air embolism (fig. 5). E.C.G. changes with fat and rape-seed embolism resembled those obtained with air embolism (fig. 6). Bilateral vagotomy did not affect the result. The interest of the rape-seed experiments is that no question of coronary-vein embolism arises, as with air embolism. The mean diameter of the rape seeds used was greater than that of the coronary veins we measured in rabbits and on no occasion did we find rape-seed emboli in structures other than the pulmonary arterial branches. Fat and starch emboli were located within the smallest pulmonary arteries and arterioles and sometimes as thin layers within capillaries. These experiments suggest that the main cause of the blood-pressure and E.C.G. disturbances is blocking of the pulmonary circulation. Interference with the outflow of blood from the coronary venous system, reflex vagal action on the heart and mechanical blockage of the coronary arteries were eliminated as factors. We would also emphasise that we obtained no evidence of

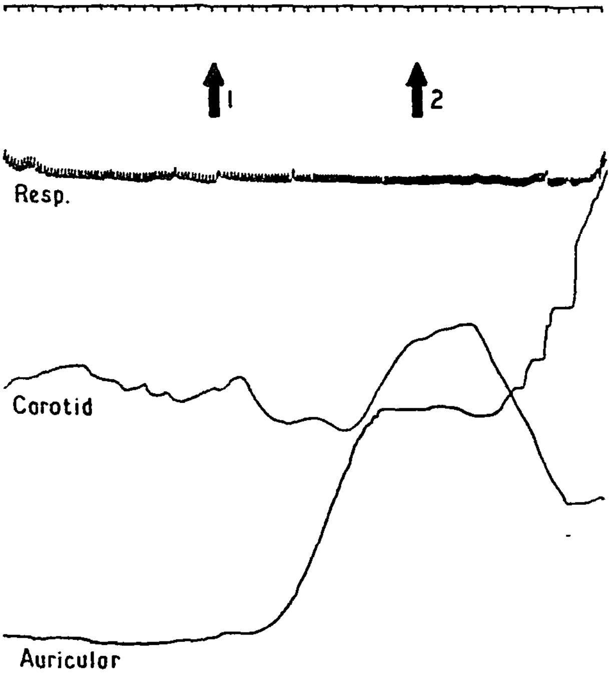

FIG. 5.—Simultaneous recordings of respiration and of carotid and right auricular pressures before and after seed embolism which was produced in 2 stages (arrows 1 and 2).

Schönbrunner, 1935, 1937 ; Radnai and Mosonyi, 1936 ; Villaret *et al.*, 1936) ; (*b*) through air bubbles in the coronary veins (von Balogh, 1939, 1941) ; (*c*) through reduced aortic pressure and increased right-heart pressure, and resulting interference with venous drainage from the myocardium.

Little doubt exists in the minds of most workers that a mechanical block between the right heart and the pulmonary outflow determines the course of air embolism. All agree that when the embolism passes a certain level of severity, determined no doubt by the size of the air bubbles, the aortic and systemic blood pressure falls as the pulmonary arterial, right ventricular, and venous pressures rise (Couty, 1875 ; Hauer, 1890 ; Jehn and Naegeli, 1918 ; Bergstrand, 1924 ; Frey, 1927, 1929 ; Singh, 1936). Our observations agree with these, and we have shown in addition, by means of cardiac catheterisation, that the right-auricular pressure increases as the systemic pressure falls. These disturbances are temporary and right themselves completely if the air embolism is not too severe or the air is introduced slowly ; at times rather large oscillations in blood pressure, in which arterial pressure drops abruptly and then recovers, suggest that a serious block in the pulmonary circulation is temporarily compensated for by the opening up of other vessels in the lungs. Singh noted similar variations in the cardiac output of dogs as the result of air embolism and explained these in the same fashion as we have done. Such phenomena are not peculiar to air embolism but are found in any form of sudden pulmonary block (Mann, 1917 ; Holden *et al.*, 1949 ; our own observations). Another factor in recovery may be solution of air bubbles, especially of their $CO_2$ content (Moore and Braselton, 1940 ; Mylks *et al.*, 1947), but in the acute cases there is little time for this to prove effective.

Right-heart failure (acute cor pulmonale) has been advocated as the main cause of death in air embolism ever since Erichsen (1844) and Cohnheim (1889) suggested that frothy blood would lead to ineffective contraction of the right ventricle. Forbes (1944), while admitting that froth would interfere with expulsion of blood from the right ventricle, stresses the obstruction of pulmonary arterioles with air, and this seems to be a fair statement of the present position. The functional disturbances are those of acute cor pulmonale secondary to the pulmonary embolism. This point of view has been well argued by McGinn and White (1935) in the case of thrombotic emboli subjected to embolectomy, and support is given by the clinical observations of Hampton and Wharton (1920), Farr and Spiegel (1929), Hall and Ettinger (1933) and the ingenious experiments on cardiac obstruction of Lundy and Woodruff (1929). So far as we know, no one has proved that frothy blood endangers cardiac contraction although there are some old experiments of Erichsen which suggest that froth hinders the flow of fluids through vessels.

From time to time a coronary or myocardial factor has been canvassed as the important cause of death in air embolism and indeed in all forms of serious pulmonary embolism, and it is this intriguing problem that has interested us most. A series of electrocardiographic studies carried out on a variety of species is summarised in table II.

TABLE II

*E.C.G. findings in air embolism*

| Species | E.C.G. findings | Attributed to | Author |
|---|---|---|---|
| RABBIT | 1. Sino-auricular tachycardia ; A.V. dissociation ; auricular flutter going on to fibrillation ; ventricular flutter going on to fibrillation | ... | Frommel, 1928 |
| | 2. Reversal of S.T. in lead I ; curve of infarct type in lead III | Block of coronary circulation especially coronary veins | Kerber, 1938 |
| | 3. Transient bundle block curve and infarct type, resembles curves in histamine collapse. Reversal of S.T. complex, curves of infarct type in lead III and regularly bundle block curves (uninfluenced by vagotomy) | Predominant injury of rt. ventricle from interference with coronary flow | Walder, 1939 |
| CAT | Increased size of P curves, ventricular fibrillation, A.V. block | Emphasises resemblance to pulmonary artery block | Krumbhaar, 1934 |
| DOG | 1. T waves inverted when pulmonary artery totally occluded. With partial occlusion, T waves at first inverted, then slowly upright | Pulmonary artery block<br>Block of rt. coronary artery | Otto, 1928-29 |
| | 2. Changes in T waves and altered ventricular complex | ... | Buchbinder and Katz, 1934 |
| | 3. Depression of S.T. segment in leads II and III. No change in lead I. Not infrequently varying degrees of A.V. block or development of nodal rhythm. Rapid return to normal with recovery | Occlusion of a coronary artery | Durant, Long and Oppenheimer, 1947 |
| | 4. No constant alteration. Depression of S.T. segment in lead III in 6/11 dogs. Inverted and diphasic T waves in lead III in 5/11. Prominent Q wave in 3/11 in leads I and III. Shifting pacemaker observed once and arterio-venous dissociation once. In fatal cases, gradual slowing of auricular rate ; no ventricular fibrillation | Coronary insufficiency from reduced aortic pressure and increased pressure in rt. heart interfering with venous drainage from myocardium | Holden *et al.*, 1949 |

Our findings are in close agreement with these. The interpretation of the E.C.G. disturbances is difficult and we were unable to decide in individual cases whether the primary factor in these disturbances was coronary or myocardial. At times we felt convinced that we were dealing with coronary insufficiency alone, for the E.C.G. pattern was similar in some respects with that obtained by ligation of the left coronary artery in dogs by Smith (1918, 1920) and Wilson *et al.* (1934), in rabbits by Taterka (1939) and in human beings with acute coronary block by Parkinson and Bedford (1927-29). In other experiments we were reminded of the patterns due to severe anoxia (Lewis and Mathison, 1910-11 ; Lewis *et al.*, 1913-14 ; Greene and Gilbert, 1921 ; Kountz and Gruber, 1929-30 ; Kountz and Hammouda, 1932 ; Rothschild and Kissin, 1933 ; Katz *et al.*, 1934 ; Opitz and Tilmann, 1936 ; Benson, 1940 ; Harris and Randall, 1944 ; Randall, 1944), or experimental collapse (Meessen, 1939 ; Taterka, 1939). We can only conclude that the E.C.G. findings reflect changing conditions in the myocardium and coronary vascular system. But we are convinced that we can carry the analysis of the coronary factor a stage further. A vagal reflex from the lungs can certainly be excluded, for bilateral vagotomy does not influence any of the phenomena of embolism. It is well known that a nervous mechanism, the Bainbridge reflex and probably other types of reflex, links pulmonary bloodflow with cardiac output, but we have obtained no evidence that these are concerned in embolism. We are also certain that the factor of coronary venous obstruction by air bubbles can be eliminated, since pressure changes and E.C.G. behaviour are identical in air embolism and rape-seed embolism. In the latter case we can exclude direct blockage of the coronary veins, since the seed emboli are much too large to enter the coronary veins. There remains the possibility of coronary flow impairment because of reduced left-heart pressure from pulmonary block and increased right-heart pressure, and it is this view we find most acceptable in explaining the E.C.G. evidence of cardiac impairment. In some experiments we could show that the E.C.G. changes appeared only when the systemic blood pressure began to fall abruptly. On several occasions we formed the impression that the myocardial factor was a serious one.

Finally, it seemed possible that cerebral ischæmia might be a factor, since a pulmonary block would lead to diminished blood flow to the brain. We have therefore recorded the E.C.G. tracings in normal rabbits before and after ligation of both common and internal carotid arteries. Much to our surprise we found no evidence of cardiac disturbance. Indeed, the rabbits seemed to be none the worse for the ligations. It would appear that the vertebral circulation suffices to keep them alive. Since the animals can tolerate such serious disturbances of their cerebral circulation, it seems unlikely that the much less severe impairment associated with pulmonary embolism can be an important factor.

## SUMMARY

Experiments are described in which air was introduced intravenously into rabbits at controlled speeds and pressures. The

pathological changes and certain functional disturbances in the cardio-vascular system due to this air embolism are discussed and contrasted with the effects of other types of embolism. Reasons are given for excluding vagal inhibition of coronary flow, obstruction of the coronary veins by air bubbles, coronary arterial embolism and primary cerebral ischæmia as factors in the circulatory upset and it is concluded that death results from pulmonary block by air bubbles. Attention is drawn to the tolerance rabbits show for sudden ligation of both carotid arteries, probably because of an efficient vertebral circulation.

We gratefully acknowledge the assistance of Drs Kenneth Harris, E. Pochin, H. H. Wolff and A. Hollman in the study of the electrocardiograms and of Mr F. J. Crew in the experiments. Expenses were met by a grant from the Graham Research Fund of the University of London.

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616 . 131—008 . 331 . 1—092 . 9 (Cuneus)

# EXPERIMENTAL PULMONARY HYPERTENSION

C. V. Harrison

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(Plate XXXI)

In a previous paper (Harrison, 1948) it was shown that repeated intravenous injections of fibrin clots into rabbits produced fibro-elastic intimal thickening in the pulmonary arteries. In addition there was some evidence that right-ventricular hypertrophy resulted from this but the evidence was equivocal because too few animals were used. It seemed desirable to repeat this work in order to ascertain firstly whether right-ventricular hypertrophy did occur, secondly whether it decreased or disappeared if the embolism was stopped and the animals allowed to recover, and thirdly whether the cardiac hypertrophy could be accounted for by simple mechanical blockage of the pulmonary arterial tree.

## Technique

The technique used was similar to that of the earlier experiment except that the injections were 2 ml. weekly instead of 1 ml. twice weekly. Forty ml. of human blood were taken aseptically (from one donor) and allowed to clot. The serum was discarded and the clot placed in a tissue homogenizer (Folley and Watson, 1948). This was allowed to run for 10 minutes at 5000 r.p.m. and effectively broke up the clot into fragments of not more than about 1 mm. diameter. The clots were washed in sterile saline till they were buff to pink in colour and then the volume was adjusted to the original 40 ml. Two ml. of this clot suspension were injected into each of 20 rabbits. A few animals died after the first injection due to faulty technique and were replaced. At the end of 20 weeks 4 rabbits had died, always soon after an injection, and it was estimated that the number of injections given had been sufficient. Six further rabbits were then killed and these together with those already dead formed group 1. The remaining ten animals were then allowed to recover for a further period of 19-22 weeks and were then killed. These formed group 2. In addition 20 healthy rabbits were killed and formed a normal control group.

In the normal control group and in group 1, the thoracic viscera were removed immediately after death and the pulmonary artery was injected with an opaque mixture of gelatine and lead oxide. Immediately after this, before the gelatine had set, the lungs were inflated to normal size via the trachea with formol saline. The whole was allowed to fix for a day or two and then the heart was removed and X-ray photographs taken of the lungs. No such injection was performed in group 2.

In all cases the auricles were removed from the ventricles and the hearts were separated into right and left according to the method of Herrmann and

Wilson (1921-22). The two ventricles were weighed separately and the weight of the right expressed as a percentage of the left (R.V./L.V. per cent.).

The details of the experiment are set out in table I.

TABLE I

*Details of experiments on pulmonary embolism*

| Animal no. | No. of weekly injections | Died or killed | Animal no. | No. of weekly injections | Duration of recovery |
|---|---|---|---|---|---|
| 19 | 12 | D | 25 | 18 | 19 weeks |
| 29 | 14 | D | 26 | 17 | 19 „ |
| 36 | 14 | D | 27 | 17 | 19 „ |
| 37 | 6 | D | 28 | 17 | 19 „ |
| 18 | 20 | K | 30 | 17 | 19 „ |
| 20 | 18 | K | 31 | 17 | 19 „ |
| 21 | 17 | K | 32 | 17 | 22 „ |
| 22 | 18 | K | 33 | 17 | 22 „ |
| 23 | 17 | K | 34 | 17 | 20 „ |
| 24 | 18 | K | 35 | 17 | 22 „ |

## RESULTS

### *Skiagram of pulmonary tree*

Satisfactory injections were obtained in 15 out of the 20 controls and fig. 1 shows the appearance of the normal pulmonary vascular tree. The main pulmonary arteries are large and regular and give off a series of wide primary branches. These frequently show localised constrictions, presumably due to muscular contraction. They in turn give rise to secondary branches which may show a few constrictions but generally have smooth outlines. Beyond these divisions the arteries are too small and too numerous for details to be visible. In the ten rabbits of group 1, three injections were technically imperfect but the remaining seven (nos. 18, 20, 21, 23, 29, 36, 37) were adequate. These did not show any recognisable embolic obstructions (fig. 2) and on the whole showed fewer constrictions in the primary branches than did the controls.

### *Arterial changes*

In the rabbits of group 1, whose pulmonary arteries were injected, intimal lesions were difficult to see because the injection mass had distended the vessel and flattened the thickened intima and also because the mass took up some stain. In the animals of group 2 this difficulty did not arise. Here the lesions had all undergone fibrosis and considerable new elastic tissue had been laid down. These lesions were exactly like those previously described and illustrated and it is unnecessary to describe them further.

### *Heart weights*

The individual weights are set out in table II. From this it will be seen that in the twenty normal controls the right ventricle expressed as a percentage of left ventricle (R.V./L.V. per cent.) had a mean value of 50·35 per cent. In the experimental group 1 (animals dying during or killed just after pulmonary embolism) the value was 67·95 per cent. In the group 2 (animals recovered for five months) the figure was 51·71 per cent. The various comparisons between these values and their statistical evaluation are set out in table III. It will be seen from this that the difference between the normal controls and group 1 is 17·6 per cent. and is highly significant but it might be argued that the animals which died of embolism were more severely harmed than those which had to be killed and that perhaps the latter did not have significant right-ventricular hypertrophy. *To overcome* this objection a comparison was made between those six animals which were killed and the controls and although the difference is less, 14·8 per cent., it is still significant. It is therefore fair to conclude

TABLE II

*Heart weights in experimental and control animals*

| Controls | | | |
|---|---|---|---|
| No | R.V. (mg) | L.V. (mg) | R.V./L.V. per cent |
| 1 | 910 | 1510 | 60 3 |
| 2 | *855* | *1580* | *54·1* |
| 3 | 750 | 1310 | 57·2 |
| 4 | 855 | 1290 | 66 3 |
| 5 | 850 | 1870 | 45 5 |
| 6 | 1285 | 2305 | 55 7 |
| 7 | 1180 | 1850 | 63 8 |
| 8 | 970 | 2320 | 41·8 |
| 9 | 1060 | 1950 | 54 4 |
| 10 | 905 | 1450 | 61·7 |
| 11 | 855 | 1860 | 45 9 |
| 12 | 655 | 1625 | 40 3 |
| 13 | 935 | 1855 | 50 4 |
| 14 | 615 | 1685 | 36 5 |
| 15 | 780 | 1680 | 46 4 |
| 16 | 825 | 1575 | 52 4 |
| 17 | 870 | 1825 | 47·7 |
| 18 | 900 | 2060 | 43 7 |
| 19 | 680 | 1990 | 34 2 |
| 20 | 770 | 1585 | 48 6 |
| | | | M = 50 35 |

| Experimental | | | | |
|---|---|---|---|---|
| *Group 1* | | | | |
| No | R.V. (mg) | L.V. (mg) | R.V./L.V. per cent | Died or killed |
| 19 | 1615 | 2370 | 68·1 | D |
| *29* | *1095* | *2310* | *84·4* | *D* |
| 36 | 1590 | 2110 | 75 4 | D |
| 37 | 1110 | 1830 | 60 7 | D |
| 18 | 1495 | 2370 | 63 1 | K |
| 20 | 1600 | 2285 | 70 0 | K |
| 21 | 1705 | 2155 | 79 1 | K |
| 22 | 1760 | 2670 | 65 9 | K |
| 23 | 1390 | 2500 | 51·6 | K |
| 24 | 1665 | 2720 | 61·2 | K |
| | | | M = 67 95 | |
| Group 2 | | | | |
| 25 | 2430 | 4140 | 58 7 | Allowed to recover for 5 months |
| 26 | 2300 | 3590 | 64·1 | |
| 27 | 1610 | 2800 | 57 5 | |
| 28 | 1150 | 2800 | 41 1 | |
| 30 | 1730 | 3725 | 46 4 | |
| 31 | 1590 | 2775 | 53 7 | |
| 32 | 1965 | 3545 | 55 4 | |
| 33 | 1380 | 2685 | 51 4 | |
| 34 | 1580 | 3570 | 44 3 | |
| 35 | 1470 | 3300 | 44 5 | |
| | | | M = 51 71 | |

that pulmonary embolism of the degree used in this experiment, does produce right-ventricular hypertrophy.

TABLE III

*Statistical analysis of the experimental and control groups*

| Comparison | Difference between means (per cent.) | Standard error of means | "t" | Degrees of freedom | P | Conclusion |
|---|---|---|---|---|---|---|
| 20 normal controls with 10 animals in group 1 | 17·60 | 3·66 | 4·81 | 28 | 0·001 | Significant difference |
| 20 normal controls with 6 animals in group 1 | 14·80 | 4·25 | 3·49 | 24 | 0·01 to 0·001 | Significant difference |
| 10 animals in group 1 with 10 animals in group 2 | 16·24 | 3·87 | 4·19 | 18 | 0·001 | Significant difference |
| 20 normal controls with 10 animals in group 2 | 1·36 | 3·09 | ... | ... | ... | No significant difference |

If now the ten animals in group 1 are compared with their ten fellows in group 2 who received similar treatment but were allowed to recover, it is seen that the difference between their right-heart ratios is 16·24 per cent. and is highly significant. This indicates that the right-heart hypertrophy disappeared when the stimulus causing it was removed.

If a similar comparison is made between the recovered rabbits in group 2 and the normal controls the difference between their mean R.V./L.V. ratios is only 1·36 per cent., which is much less than its own standard error and not at all significant. This suggests that the disappearance of right-heart hypertrophy was complete.

## DISCUSSION

This experiment shows that repeated showers of small pulmonary emboli (1 mm. diam.) produce right-ventricular hypertrophy and presumably do so by raising the blood pressure in the pulmonary circulation. This rise of pressure lasts only as long as the emboli continue to impact and when this ceases the right-ventricular hypertrophy disappears completely. Injection of the pulmonary arteries failed to show any apparent obstruction to the pulmonary circulation, which implies that the rise of pulmonary blood pressure was not the result of simple mechanical blockage. This is in line with previous experimental and human observations. The operation of pneumonectomy removes one-half of the total pulmonary circulation yet it does not give rise to pulmonary hypertension (Brenner, 1935); Holden *et al.* (1949) produced massive pulmonary embolism in dogs and found that it was necessary to obstruct 70 per cent. of the pulmonary circulation before the animals died; degrees of embolism

## EXPERIMENTAL PULMONARY HYPERTENSION

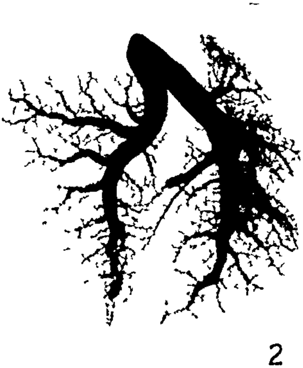

FIG. 1.—Control rabbit. Skiagram of injected pulmonary arteries showing the normal pattern.

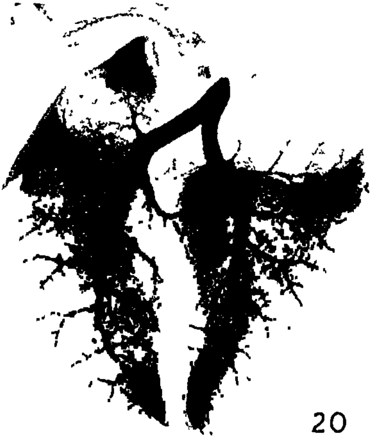

FIG. 2.—Experimental rabbit no. 20. Injected pulmonary arteries showing absence of any visible occlusion.

less than this caused only transitory circulatory changes to which the dogs rapidly became adjusted. Similarly Haynes *et al.* (1947) showed that occlusion of a main pulmonary artery in dogs by an inflatable balloon did not cause a change in the pulmonary blood pressure. On the other hand, they found that the injection of a quantity of minute emboli caused a rise of pulmonary pressure and other acute circulatory changes. From these observations it appears that two different mechanisms are at work according to whether large or small emboli impact. If large emboli become impacted the result is a simple mechanical obstruction which must be massive to cause death. If, on the other hand, a shower of minute emboli becomes impacted there is a rise of pulmonary pressure which is disproportionate to the proportion of the vascular bed obstructed, and some other mechanism has to be invoked to explain it. In the greater circulation there is evidence that the impaction of an embolus in a muscular artery or even in an elastic artery causes spasm both at site of impaction and in the adjacent branches (Allen *et al.*, 1946; Learmonth, 1948). In the lesser circulation the same phenomenon has not been demonstrated with certainty, but Sherf and Boyd (1948) suggest that it probably does occur and offer indirect evidence in favour of this view. Daly *et al.* (1947) have shown that in dogs a vasomotor mechanism exists and is capable of causing a rise of pulmonary blood pressure.

From these various observations it seems reasonable to assume that the impaction of small emboli in the finer branches of the pulmonary arteries produces a reflex spasm which causes a rise of pulmonary blood pressure in excess of that caused by the mechanical blockage, and further, that this spasm is not induced or is of minor degree if the occlusion occurs in the larger pulmonary arteries and that here mechanical obstruction is the dominant factor. The results of the present experiment add further evidence in support of this hypothesis so far as it concerns the smaller type of vessel.

## SUMMARY

Twenty rabbits were subjected to repeated showers of minute pulmonary emboli consisting of fibrin clot. Ten of these, killed immediately after embolisation, had a significant degree of right-ventricular hypertrophy, but post-mortem injection and X-ray of their pulmonary arteries did not show any vascular occlusion. Ten rabbits that were allowed to recover for 20 weeks after embolisation had no right ventricular hypertrophy. It was concluded that minute pulmonary emboli cause a rise of pulmonary blood pressure by inducing vascular spasm.

I am indebted to Dr D. A. Mitchison for his help with the statistical analysis, to Dr Duncan White for the skiagrams and to Mr E. V. Willmott for the photographs.

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616 . 13—002 . 2—018 . 91

# INTIMAL VASCULARISATION AND ATHEROSCLEROSIS

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(Plates XXXII-XXXV)

The history of the study of atherosclerosis in the past falls rather sharply into two phases. The first, which was characterised by an exhaustive analysis of the morbid histology, led to the isolation of atherosclerosis as a separate entity and to the formulation of a great variety of hypotheses as to its ætiology and pathogenesis. Although the hopes of arriving at a full understanding of the causes of this disease by a minute analysis of its microscopical features were not fulfilled, it was during this phase that the basic facts were accumulated on which all later work rests and must continue to rest. This achievement was largely the work of German pathologists in the second half of the 19th century and is linked with the names of Rokitansky, Virchow, Thoma, Marchand and Jores.

The succeeding phase, which may be referred to as the biochemical and experimental one, dates from 1913, the year in which Anitschkow succeeded in inducing lipoid lesions in the aorta of rabbits by raising the serum-cholesterol level. The theoretical implications of this discovery, and even more the fact that it provided a comparatively simple and reliable experimental method, led to a widespread and intensive study of the relation of cholesterol metabolism to atherosclerosis, and in this new phase Anglo-Saxon investigators contributed notably. It is probably fair to state that this phase also has failed to provide a satisfactory concept of the ætiology of human atherosclerosis, but inevitably it has increased our understanding not only of this disease but also of cholesterol metabolism generally, both normal and abnormal.

Meanwhile, in 1938, by the publication of their book, *The biology of arteriosclerosis*, Winternitz and his co-workers opened up a new approach which, together with the interesting observations of Duguid (1946), may well mark the beginning of a new stage in the study of this important disease. Winternitz's contribution consisted in a careful and thorough demonstration of the phenomenon of intimal vascularisation, a phenomenon which, although recognised by the early German investigators, had never been accorded its proper importance in the genesis of the late complications of atherosclerosis. Winternitz was able not only to describe and to illustrate beautifully the anatomy of intimal blood channels but also to demonstrate convincingly that a study of the vascular disturbances of atherosclerotic plaques provided the key to an understanding of many of the most striking features of the later phases of the disease. Indeed, he went further and claimed for intimal vascularisation a decisive part in the inception of atherosclerotic lesions but in this we may not be able to follow him. The work of Duguid also has its roots in the observations of 19th century pathologists. The great Rokitansky (1852) was of the opinion that atherosclerosis was caused by an excessive accretion and deposition of an inner vascular layer from the blood stream. Under the impact of Virchow's opinions, however, Rokitansky largely modified his view and in 1856 regarded it as merely a helpful nomenclatural concept (pp. 313-14). It remained for Duguid to demonstrate clearly that mural thrombi do indeed become incorporated into the intima in such a fashion as to be indistinguishable from atherosclerotic thickenings.

At first sight these two individual contributions to the problem of atherosclerosis may seem rather unrelated, and it is one of the objects of this paper to demonstrate the connection between these two phenomena and to explain why one cannot be understood without the other.

As is usual, these important advances in our knowledge were gained by the employment of special techniques, injection and clearing in the one case and serial section in the other. In order that these acquisitions may become common property of pathologists and so the basis for further advances, it is desirable that we should learn to see them in ordinary routine sections. It is a common and important development that facts which at first can be elucidated clearly only by a specialist technique can thereafter be appreciated by simpler methods. It is the further aim of this paper to show how, based on the work of Winternitz, Duguid and others, the important features of intimal vascularisation and of thrombogenic atherosclerosis can be recognised and studied in single routine sections.

## *Material and methods*

The material for this study, which was derived from routine autopsies at the Royal Infirmary, Edinburgh, consisted of 300 aortas taken in their entirety and sections from 100 hearts, including the first inch of the anterior descending branch of the left coronary artery. The specimens were fixed in 10 per cent. formol and blocks from suitable portions were cut in series by the paraffin method and occasionally with the freezing microtome. The sections were stained with hæmatoxylin and eosin. No fixed system was followed in the examination of the serial sections, which were sometimes consecutive, sometimes at intervals of three or four, or singly, depending on the point under investigation, the most commonly employed thickness of the sections being 10 $\mu$.

### *The growing intima of human arteries is an avascular structure and remains so in the vast majority of instances throughout life*

This important and easily verifiable fact has been vaguely challenged by the work of Winternitz and his associates and it may therefore be of some importance to re-emphasise it. The presence of a vascular network in the adventitia and media of arteries is an established fact of anatomy and histology. It has also been known, at least since the work of Rokitansky (1856, pp. 310-11) that old atherosclerotic plaques become vascularised. It is very different with regard to the normal intima. All observers have consistently failed to discover a vascularised plexus in the subendothelial space. Winternitz conveys the impression that he thinks the normal intima to be vascularised, yet a careful perusal of his book fails to reveal any evidence for such a view. The result of his investigations in this respect is best summed up in his own words (p. 111). " The lack of demonstrable vessels in the inner media and intima . . . has not been absolute ". This double negative refers to the fact that on a few occasions he has been able to open up isolated " potential " blood channels. This was accomplished by an injection technique employing pressures up to 1500 mm. of mercury, *i.e.* ten times the pressure prevailing in the aorta during life. Manifestly, it is misleading to try to establish normal channels of blood flow by such methods, even apart from the artefacts (" sinusoids ") which are thus created. Even so, Winternitz has at best been able to demonstrate the very occasional occurrence of isolated intimal blood channels and it is to be deplored that he creates the impression of believing in vascularity of the human arterial intima as a common and normal phenomenon. This criticism has to be expressed in clear terms, as the very excellence and importance of the rest of his findings might create a danger of this untenable concept being accepted along with them. That the normal intima should be an avascular structure becomes understandable when we examine the physiology and mode of nutrition of the subendothelial layer. Having regard to the high intra-arterial pressure and to the permeability of the endothelial lining it cannot be doubted that the innermost layer of the arterial wall derives its nutriment directly from the main lumen. This permeation of plasma constituents through the endothelium was postulated by Virchow and has been demonstrated by, among others, Petroff (1922) and Duff (1932). The depth of tissue which can thus be directly nourished by filtration from the main lumen depends on the height of the filtration pressure and is therefore greater in the large arteries than in other blood vessels. In normal arteries this depth corresponds approximately to that of the intima plus the inner third of the media. We might, on this basis, distinguish a physiological intima, meaning that part of any blood vessel which is nourished directly from the main lumen.

An adequately nourished tissue provides no stimulus for capillary growth and the arterial intima will therefore remain an avascular structure while its depth remains within the radius of trans-endothelial filtration. The simplest way of understanding this matter is to consider the endothelial tube which lines the main arteries as the capillary bed of the subendothelial tissues different from other capillary beds only in the higher pressure at which it delivers its filtrate. This arrangement becomes inadequate if the intima grows beyond a certain thickness or density and under such conditions the intima can form or receive capillaries.

### *Intimal vascularisation is a phenomenon secondary to excessive intimal growth and the intimal depth at which vascular networks will first make their appearance varies in each artery with the height of the intra-arterial pressure*

In an examination of 300 aortas (Geiringer, 1949) and of 100 coronary arteries (Geiringer, 1951) the critical depth beyond which vascularisation makes its appearance was found to be approximately 0·5 mm. for the aorta and approximately 0·35 mm. for the first inch of the anterior descending branch of the left coronary artery. Vascularisation will occur in any stretch of arterial intima which exceeds the critical depth, whether the area concerned be the seat of atheromatous lesions or not. It is true that a thickening beyond the critical depth is usually associated with atheroma, but where such thickenings happen to be of a purely fibrous type, vascular channels can nevertheless be found. They are, in these instances, not as numerous as in an atheromatous plaque of equal thickness, but it is of fundamental importance to realise that intimal vascularisation is a function of intimal thickness and not of atherosclerosis. The examination of single routine hæmatoxylin and eosin-stained sections for intimal vascularisation does not, of course, reveal the intricate network which can be outlined by the injection technique. Nevertheless, the existence of intimal blood channels can almost always be demonstrated in intimas of more than critical thickness, and with careful search they will be found in well over 90 per cent. of cases in the examination of a single slide. On the other hand, in intimas of less than critical depth they are never found.

Three types of intimal plexus can be distinguished, namely transmedial vascularisation, vascularisation from the lumen, and a combination of the two.

### *Transmedial vascularisation*

It is a common experience that the normal vascularity of the thin-walled arteries of young subjects is confined to the adventitia and that with advancing age and increasing thickness, the outer two-thirds of the media also take part in this vascularity. If the intima grows even thicker, vessels begin to appear in the inner third of the media and eventually penetrate the internal elastic lamina,

INTIMAL VASCULARISATION

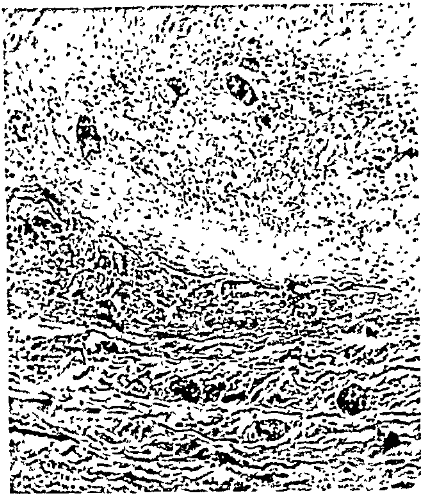

FIG. 1.—Transmedial vascularisation of the intima, showing disruptive effect on the media. Coronary artery. ×100.

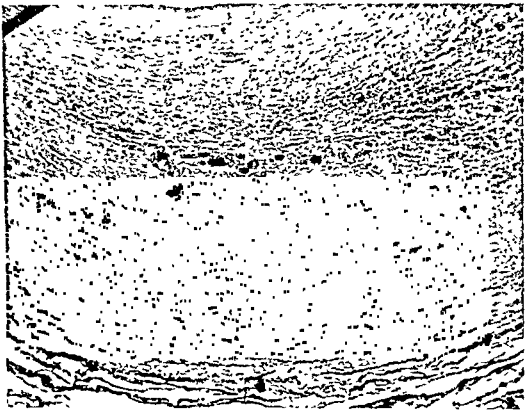

FIG. 2.—Thrombogenic vascularisation of the intima. The intimal channels run at the critical depth. Coronary artery. ×50.

## INTIMAL VASCULARISATION

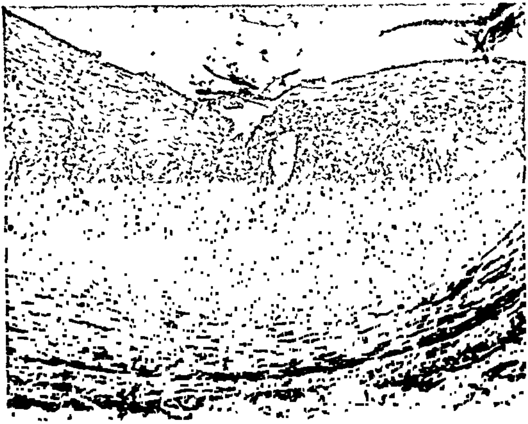

FIG. 3.—Giant capillary joining the lumen of a coronary artery. ×85.

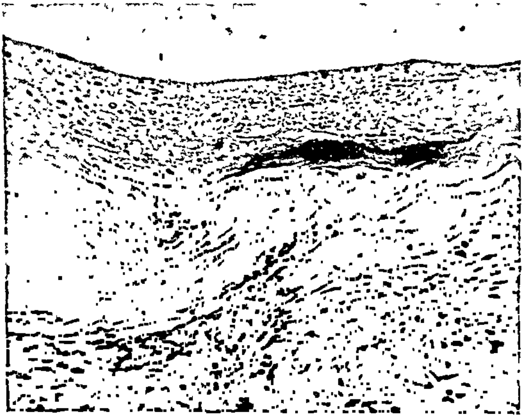

FIG. 4.—Thrombogenic vascularisation of the intima. A capillary is seen proceeding obliquely towards the lumen. Coronary artery. ×190.

INTIMAL VASCULARISATION

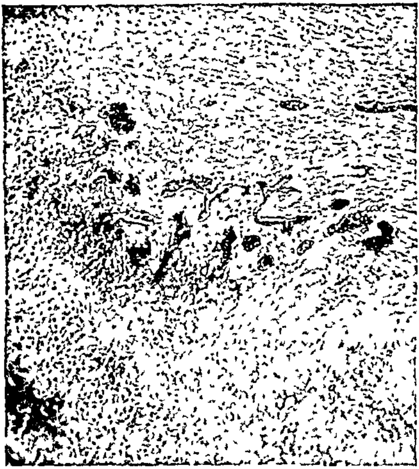

FIG 5.—Thrombogenic vascularisation of intima showing bizarre outlines of blood spaces. Coronary artery. ×85.

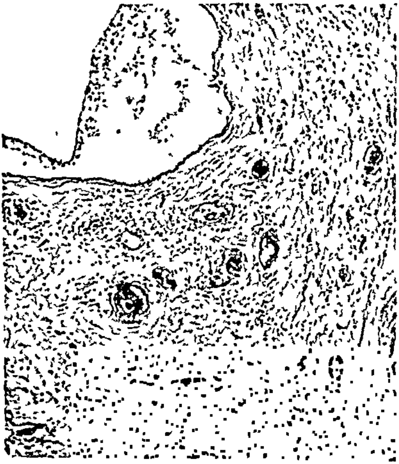

FIG 6.—Thrombogenic intimal vascularisation Excessive vascularity of not fully organised thrombus Coronary artery. ×100

INTIMAL VASCULARISATION

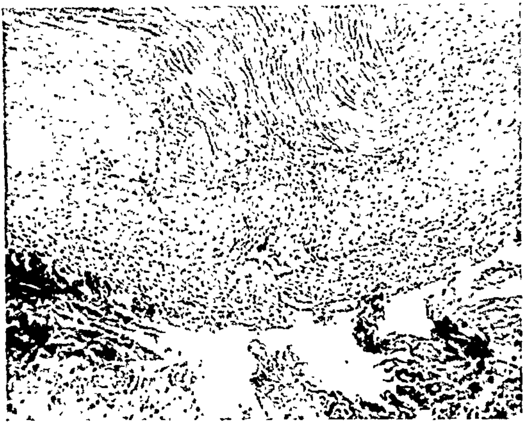

FIG. 7.—Transmedial vascularisation at the base of an avascular and partly necrotic thrombogenic intimal thickening. Coronary artery. ×80.

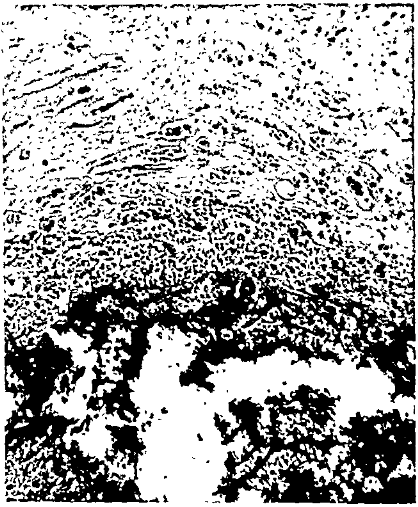

FIG. 8.—Extension of thrombic capillaries into underlying intima. Coronary artery. ×210.

thus becoming intimal. This process was described in some detail by Wolkoff (1929). Microscopic study of sections showing this trans-medial type of intimal vascularity creates the strong impression that such abnormal permeation and disruption of the medial coat by blood vessels cannot take place without interference with medial function (fig. 1). That increased vascularity might weaken the media was pointed out by Koester as early as 1875 (quoted by Jores, 1903), and it would appear probable that we are dealing here with one of the vicious circles in which pathology abounds ; for while vascularisation of the media seems to be caused by intimal thickening, it cannot be doubted that any resulting weakening of the media would be a stimulus to further intimal hyperplasia. The same applies to cases of primary medial disease where the compensatory intimal hyperplasia will provide a stimulus to medial vascularisation and thus to its further weakening.

These vessels, which are presumably formed by progressive sprouting of capillaries towards an area of relative ischæmia, retain their capillary structure, *i.e.* they rarely acquire a media or adventitia, though their calibre is quite often of arteriolar dimensions. When they reach the intima they penetrate it until they have reached a certain critical distance from the lumen. They then give off terminal branches which tend to remain at roughly the same critical distance from the lumen. This type of intimal vascularisation is found most often where the intima has thickened by slow degrees and in a more of less concentric fashion. It is, so to speak, the " normal " method by which the intima acquires an adventitious blood supply, since it is derived from the normal peri-arterial plexus. It is seen most frequently in the fibrous hyperplasia without marked atheroma so often encountered in the coronary arteries of aged men. Plaques of atheroma also may show this variety, but more often display the second type of intimal vascularisation.

### *Vascularisation from the lumen*

Woodruff (1926) has noticed the occasional occurrence of blood channels starting from the main lumen and traversing the intima in the ascending aorta of the dog and Winternitz (p. 27) has seen similar appearances in the aorta of a cow and thinks that channels of this kind can also be found in normal arteries of man. There is, however, no support in the literature for such a belief, and in the present series of human aortas and coronary arteries, direct intimal vascularisation from the main lumen was never seen in otherwise healthy vessels. It may be accepted that such channels, if they occur at all in normal human arteries, must be rare indeed. Moreover, the above-cited cases refer to channels which traverse the intima without giving rise to branches. On the other hand, a type of intimal vascularisation in which the supplying vessel can be traced from the main lumen of the artery is quite commonly seen in association with atheromatous plaques, especially where these are eccentric and exuberant. Duguid's demonstration of the thrombogenic nature of many of these plaques has thrown a flood of light upon this problem. A study of the fate of mural thrombi with special reference to their vascularity leads to the following conclusions. Vascularisation from the lumen is met with in plaques of thrombotic origin and may indeed be taken as evidence of the thrombotic origin of such a plaque. This type of intimal blood supply represents the remnants of the vascularisation which any mural thrombus undergoes in the course of its organisation. If the organised thrombotic material plus the underlying original intima does not exceed the critical depth for the artery in question, then the thrombic capillary plexus will, in course of time, completely disappear, leaving an avascular intima behind. Where, however, as frequently happens, the added mass of the thrombus creates an intima too thick to be nourished by filtration from the main lumen, a portion of the thrombic plexus will remain patent and spread itself in the intima at roughly the critical distance from the lumen (fig. 2). The supplying giant capillary will in these cases join the lumen, as is natural in view of its origin (fig. 3). In single routine sections, it is not often possible to see the orifice into the lumen of the supplying vessel. Two criteria may then be employed to recognise this second type of intimal vascularisation. First, it will often be possible to see portions of the blood channel and to follow it for some distance in the direction of the lumen (fig. 4); second, the endothelium-lined blood spaces in the intima will often be found excessively large and of bizarre outline (fig. 5). This is a characteristic of thrombogenic vascularisation and is the result of secondary endothelialisation of the irregular interstices of the shrinking thrombus (Heuking and Thoma, 1887). Intimal vascularisation from the lumen is found much more commonly in the aorta than in the coronary arteries.

This thrombogenic method of intimal vascularisation complicates the study of this question by presenting an obvious source of fallacies. It takes a considerable time for a mural thrombus to reach the quiescent stage of full organisation and until then it will show greater vascularity than is in accordance with the above-described theory of critical depth. In the earlier stages when it is readily recognisable as a mural thrombus, this does not create any confusion, but later, when it has become to a large extent incorporated in the intima, this excessive vascularity may confuse the issue unless one realises the thrombotic nature of the intimal thickening (fig. 6). Conversely, retention of a sufficient vascular supply often fails to occur and the incorporated thrombus then presents as an avascular addition to the intima with transmedial vascularisation on its deep aspect and necrosis in its centre (fig. 7).

These two types of intimal vascularisation thus provide a ready means by which to distinguish two types of intimal growth :—(1)

transmedial vascularisation, with an intima which has gradually, by steady growth or continuous accretion, progressed beyond its critical thickness, and (2) vascularisation from the lumen, which is witness to a sudden and catastrophic growth in thickness of the intima by mural thrombosis.

### *Combined form*

It is not surprising that these two forms of intimal vascularisation are often found in association in the same stretch of artery. Where they coexist, they can usually be shown by serial section to have established anastomoses. Thrombosis, no doubt, often occurs over an intima which already possesses a transmedial blood supply, but it must be emphasised that mural thrombosis over an avascular intima renders the underlying tissues ischæmic and is itself a potent stimulus to proliferation of adventitial capillaries towards the intima. At the same time, vascularisation of the thrombus can often be seen extending into the underlying intima (fig. 8). In this way, the result of mural thrombosis is not simply a thrombogenic vascularisation of the intima but, more often perhaps, the establishment of a combined network linking the main lumen at a number of points with the vasa vasorum. In Winternitz's book there are many beautiful illustrations of this form of vascularity, but the pathologist working with only one or two thin and uninjected sections will usually have to be content with suspecting its existence when he encounters features of transmedial vascularisation associated with others suggesting vascularisation from the lumen.

### *Fate of the vascularised intima*

Secondarily evolved blood supplies occurring in pathological new formations are notoriously unstable and subject to interference. They are built up haphazard in response to local need and lack the safeguards which characterise the more hereditarily determined plexuses of normal organs. Often they run in abnormal tissues which do not provide them with sufficient support. They lack good collateral connections and shunts and are built on a marginal basis. The component vessels are often of the giant capillary type, *i.e.* with walls inadequate for the volume of blood which they carry. An intima depending on such a precarious blood supply is prone to secondary disturbances which, because of the proximity of the arterial lumen, may not be confined to this tissue but have serious local and distant repercussions.

### *Necrosis*

Intimal necrosis may result from failure of an incorporated mural thrombus to retain or acquire a sufficient blood supply. The causes of such failure are obscure. When, on the other hand, adequate vascularisation has occurred, there are probably two mechanisms by which necrosis may be induced. The supplying vessel may become blocked by thrombosis, as was demonstrated by Winternitz, or infarction may result from sudden lowering of blood pressure and consequent failure of supply to this marginally nourished tissue. Where the hypertrophied and vascularised intima is of a purely fibrous character, the resulting infarct will tend to have the characteristics of hyaline necrosis, whereas infarction of an atherosclerotic focus will result in classical "atheroma", *i.e.* a collection of dead connective tissue, disintegrating cholesterol esters and crystals of free cholesterol. Calcium also will appear in increased amount in these necrotic atherosclerotic plaques. Since intimal necrosis is almost invariably a consequence of a disturbed secondary blood supply it is never seen in plaques or stretches of intima which are below the critical depth. In the past, it has been regarded as paradoxical that very big plaques may sometimes escape necrosis, while comparatively small ones show this change to a marked degree. This we can now readily understand. The occurrence of necrosis is determined by the breakdown *of the adventitious* blood *supply and this in turn is a* result of factors which may have no relation to the size of the plaque. Winternitz has stressed the importance of intimal hæmorrhage as an independent accident which often befalls these vascularised plaques. Such hæmorrhages, however, are never seen in the absence of necrosis and are therefore probably more often the consequence of ischæmic infarction, the disintegration of the supporting ground substance being a likely cause of capillary rupture. We may in these cases justifiably speak of hæmorrhagic infarction.

### *Local repercussions*

There is a tendency for red thrombi to form over infarcted plaques. Some authors consider this to be a result of the ulceration of these plaques, but microscopic study leaves no doubt that red thrombi also form over well-covered necrotic plaques. The mechanism in these cases is probably a release of thromboplastic substances from the necrotic material inside the plaque. It is a similar mechanism to that responsible for the formation of mural thrombus in myocardial infarction.

This tendency to clot formation over necrotic plaques is of relatively little importance in the aorta. An application of these facts to smaller vessels, however, and especially to the coronary arteries, is of great interest. Coronary thrombosis occurs usually over necrotic atheromatous plaques, and has been shown to be often associated with intra-plaque hæmorrhages (Paterson, 1936). Intra-plaque hæmorrhages can also be found in non-occluded coronaries.

The sequence of events may be constructed variously :

1. The plaque, which as Duguid has shown, may itself be largely a product of thrombosis, becomes vascularised. Extension of atheroma, capillary thrombosis, a sudden lowering of

systemic arterial pressure, or a spastic narrowing of the artery, interferes with its blood supply. It becomes infarcted and a thrombus forms over it.

2. A thrombus occurring independently of intra-plaque changes occludes the coronary artery and at the same time interferes with the blood supply of the plaque, which becomes infarcted.

The first sequence is probably the more common, so that it would appear that coronary (plaque) infarction is the most frequent cause of coronary thrombosis. Summarising in general terms we may say that plaque infarction often causes thrombosis and that thrombosis is one important cause of plaque infarction.

### *Distant repercussions*

Three phenomena may be mentioned here, one of which is certainly, one probably and one possibly related to intimal necrosis. (*a*) Embolic dissemination of necrotic material or of detached mural thrombi. Both of these accidents are known to occur with considerable frequency (Meyer, 1947 ; Zak and Elias, 1949). (*b*) Increased clotting tendency of the blood due to the release into the circulation of thromboplastic material from the necrotic focus. The amount of such material released from a single plaque is probably not sufficient to cause a significant alteration in the clotting tendency of the blood, but when wholesale necrosis of many big plaques has occurred, as can sometimes be seen in the aorta, this may become an important factor. In the same way the increased clotting tendency observed after myocardial infarction (Wright, 1946) can probably be accounted for by the release of thromboplastic material from the dying heart muscle. (*c*) The exact physiology of collateral circulation is still very poorly understood, but it would seem possible on present knowledge that the irritation by a necrotic focus in one part of an artery might be the cause of disturbed function and, more especially, of spasms in related parts of the arterial tree (Meiners, 1950).

### *Clinical significance*

Living atherosclerotic thickenings are rarely a source of trouble. The only clinical effect which we may ascribe with any degree of confidence to the existence of non-necrotic atherosclerosis is an occasional significant stenosis of one of the main ostia, especially those of the renal or coronary arteries. The vast majority of vascular accidents due to atherosclerosis occur in relation to necrotic plaques and, as we have seen, intimal vascularisation is the forerunner of intimal necrosis. The new phase in the study of atherosclerosis thus concentrates attention on the feature which confers upon this lesion its claim to one of the most important killing conditions. A full understanding of intimal vascularisation is the prerequisite of a rational therapeutic approach to the question of late atherosclerosis. This latest phase is even less likely than the preceding ones to unveil the mysteries of the ætiology of atherosclerosis and of the pathogenesis of its early lesions, but by giving a pathological basis to the trials of anticoagulants it promises already to yield some practical benefit to the sufferers from this widespread affliction and this, after all, must remain the main object of pathological research.

## SUMMARY

The study of 300 human aortas and of 100 coronary arteries leads to the following conclusions :—

1. The normal arterial intima is an avascular structure.
2. It acquires a secondary blood supply if it grows beyond a certain critical thickness.
3. This critical thickness, which varies with each artery, is about 0·5 mm. in the aorta and 0·35 mm. in the proximal part of the anterior descending branch of the left coronary.
4. Intimal vascularisation occurs either through an extension of the adventitial plexus into and through the media or by retention of the vascular network of organising mural thrombi. When both processes occur in the same vessel, anastomoses usually form.
5. This adventitious blood supply of the intima is liable to interference, and most of the clinical effects of atherosclerosis can be traced to the resulting ischæmic necrosis of the intima.
6. These phenomena can be appreciated and studied in uninjected routine sections.

I wish to thank Mr T. C. Dodds of the Pathology Department of Edinburgh University who prepared the micro-photographs. The material for this paper was derived from routine necropsies at the Royal Infirmary, Edinburgh.

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# ŒDEMA AND CAPILLARY ANOXIA

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(PLATE XXXVI)

CAPILLARY anoxia is stressed as an important factor in the causation of œdema and effusions in most monographs and reviews which refer to the subject (Starling, 1909; Adolph, 1933; Drinker and Field, 1933; Peters, 1935; Drinker, 1938; Van Liere, 1942; Davson and Danielli, 1943; Landis, 1946; Himsworth, 1947). In several original contributions to the literature on œdema (Bainbridge, 1906; Bolton, 1915-16; Barbour and Hamilton, 1925; Adolph and Lepore, 1930-31; Diamond *et al.*, 1932; Clifford, 1941; Homans, 1943; Trowell, 1946-47) the effect of anoxia on the capillaries is used to explain the production of various types of œdema, effusions, or increased lymph flow, which are not readily explicable in other ways, though there is no direct support for the anoxia hypothesis in these studies.

Most of the direct evidence on the subject comes from studies of the effects of general rather than local anoxia. While some of the studies suggest that capillary anoxia is a cause of œdema, many do not. The direct evidence will be discussed later.

The purpose of the experiments to be described was to obtain further information concerning the role of local capillary anoxia in the production of œdema.

## METHODS

*Principle.* The principle of the method used was to perfuse one limb of an intact animal with deoxygenated blood under conditions as nearly physiological as possible. In its simplest form this was found to be impracticable, as no satisfactory method could be devised for artificial deoxygenation of the blood without gross interference with the circulation. It was decided, therefore, to utilise normal venous blood as the perfusate.

One hind limb, the perfused limb, was perfused via its femoral artery, with blood flowing from the femoral vein of the other hind limb, the donor limb. The pressure of the venous blood delivered to the perfused artery was increased to arterial pressure by means of a perfusion pump. After supplying the perfused limb, the twice-used blood returned via the femoral vein of that limb to the heart for natural re-circulation through the entire body of the animal. Dogs were chosen for the experiments as the cannulations and other manipulations would have been too difficult in smaller animals.

*Perfusion apparatus.* A modification of the Dale and Schuster system (1927-28) was used to maintain the perfusion (fig. 1).

Blood from the cannulated femoral vein of the donor limb passed through polythene tubing (A) into a glass reservoir (B), whence it was drawn through a simple non-return valve (C) into a glass chamber (D) by the contraction of a rubber sac (E) connected to a Dale-Schuster diaphragm pump. At each expansion of sac (E) blood was forced through a second non-return valve (F) into the polythene tubing (G) and thence into one arm of a Y-shaped cannula (H), reaching the perfused limb by the stem of this cannula. The other arm of the cannula communicated with a by-pass (I) by which blood could return to the reservoir (B). This by-pass was controlled by a resistance (J) which consisted of thin rubber tubing sealed inside a glass tube connected to a manometer. By varying the pressure outside the thin-walled tubing of the resistance, a greater or lesser proportion of the artificial circulation could be returned to the reservoir, and in this way the quantity of blood entering the perfused limb could be regulated. During an experiment the pressure in J was adjusted as required to maintain a constant blood level in the reservoir (B). As long as this constant level was maintained, it is clear that blood must have been leaving the perfusion system for the perfused limb at the same rate as the blood was entering the perfusion reservoir from the donor limb.

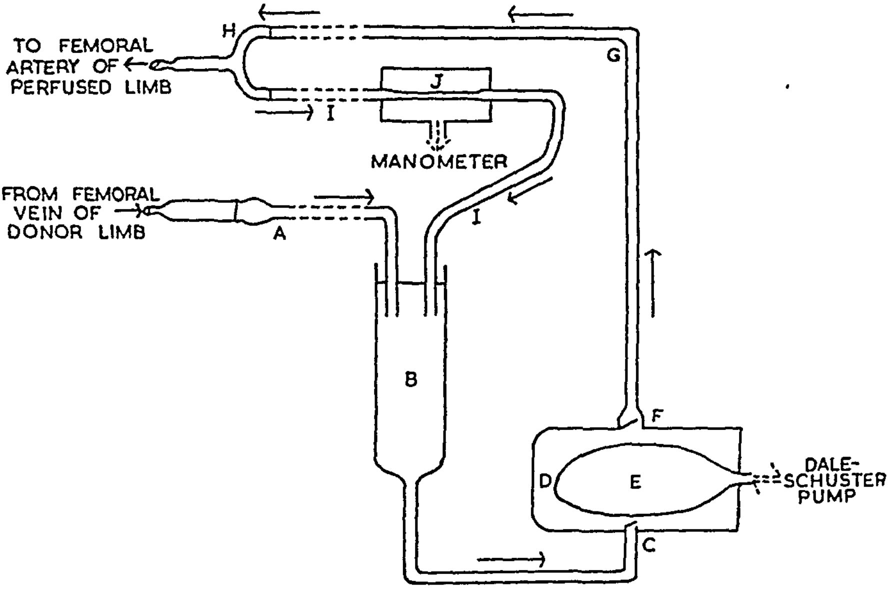
H
TO FEMORAL ARTERY OF PERFUSED LIMB
G
J
I
MANOMETER
FROM FEMORAL VEIN OF DONOR LIMB
A
I
B
F
D
E
DALE-SCHUSTER PUMP
C

FIG. 1.—Perfusion system.

In practice a double Dale-Schuster pump with paired valve chambers was used to give greater pumping efficiency at the rapid pulse rates usually encountered.

The entire apparatus apart from the cannulæ and attached polythene tubing was immersed in a water bath kept at a temperature about ½° C. higher than the rectal temperature of the dog. Polythene is a poor conductor of heat and this helped to prevent heat loss by the blood circulating in that part of the perfusion system lying between the water-bath and the dog. During a perfusion, blood circulated rapidly from the pump through the arms of the Y-shaped cannula and back through the by-pass. As a result, the temperature of the blood in the arms of the cannula remained at water-bath temperature. The blood leaving the system by the stem of the cannula had only a very short distance to travel to enter the perfused limb : thus the perfused blood was for all practical purposes at the same temperature as the water-bath. In actual observations the temperature of the perfused blood was always found to be within 1° C. of the temperature of the dog and usually within 0·5° C.

All glass tubing connections and both cannulæ were coated with a water repellent (" Teddol "—British Thomson-Houston Co., Ltd.) in order to minimise the formation of the small fibrin clots which are prone to develop in perfusion pumps even after the most efficient heparinisation of the blood. Between experiments all apparatus was thoroughly cleaned and sterilised and the " Teddol " re-applied.

*Technique.* The dog was anæsthetised with either pentothal and endotracheal ether, or with Nembutal; atropine pre-medication was usually used. Aseptic precautions were maintained throughout the experiment. The femoral vein of the donor limb, usually the right, and femoral artery of the perfused limb, usually the left, were exposed and prepared for cannulation. When capillary oozing from the operation wounds had ceased, the animal was given 1000 I.U. of heparin per kg. intravenously and left for 10 minutes for the heparin to take effect. During this time the pumping apparatus, previously filled with 120 ml. of sterile physiological saline, was also treated with heparin and the temperature of the water-bath was regulated. Any oozing of blood from the wound sites during the experiment was reduced by the topical application of alginate wool and solution (" Calgitex "—Thackray, Ltd.). At the end of the 10-minute interval, the femoral vein of the donor limb was ligated and a large-bore venous cannula inserted below the ligature. The femoral artery of the perfused limb was then ligated and the stem of the Y-shaped cannula inserted distally. The clamps on the various polythene tubes were removed and the perfusion started. The revolutions of the Dale-Schuster pump were synchronised with the pulse rate of the dog.

The saline which had been used to fill the perfusion apparatus initially was the first fluid to reach the perfused limb but was rapidly replaced by venous blood from the donor limb flowing through the natural re-circulation through the entire body of the animal. Dogs were chosen for the experiments as the cannulations and other manipulations would have been too difficult in smaller animals.

*Perfusion apparatus.* A modification of the Dale and Schuster system (1927-28) was used to maintain the perfusion (fig. 1).

Blood from the cannulated femoral vein of the donor limb passed through polythene tubing (A) into a glass reservoir (B), whence it was drawn through a simple non-return valve (C) into a glass chamber (D) by the contraction of a rubber sac (E) connected to a Dale-Schuster diaphragm pump. At each expansion of sac (E) blood was forced through a second non-return valve (F) into the polythene tubing (G) and thence into one arm of a Y-shaped cannula (H), reaching the perfused limb by the stem of this cannula. The other arm of the cannula communicated with a by-pass (I) by which blood could return to the reservoir (B). This by-pass was controlled by a resistance (J) which consisted of thin rubber tubing sealed inside a glass tube connected to a manometer. By varying the pressure outside the thin-walled tubing of the resistance, a greater or lesser proportion of the artificial circulation could be returned to the reservoir, and in this way the quantity of blood entering the perfused limb could be regulated. During an experiment the pressure in J was adjusted as required to maintain a constant blood level in the reservoir (B). As long as this constant level was maintained, it is clear that blood must have been leaving the perfusion system for the perfused limb at the same rate as the blood was entering the perfusion reservoir from the donor limb.

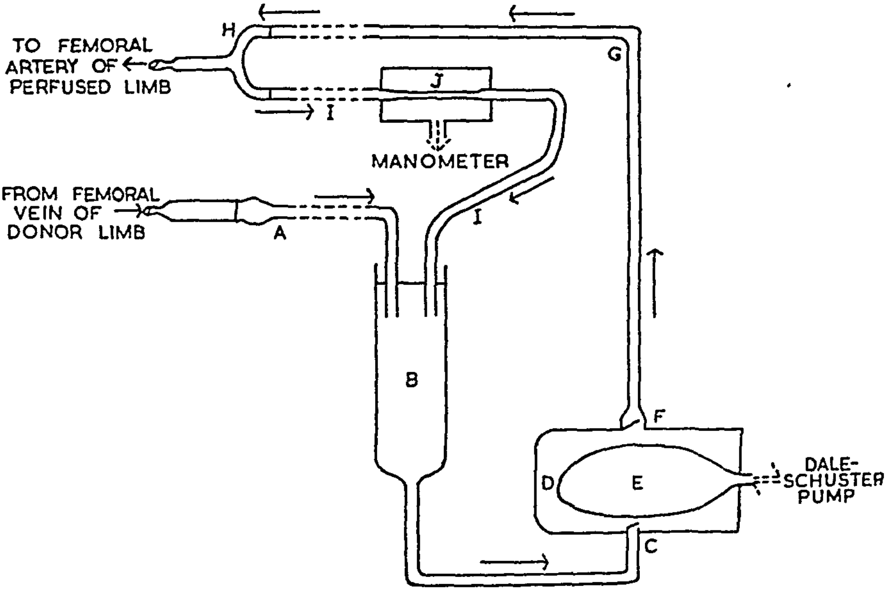

Fig. 1.—Perfusion system.

The oxygen content of the blood issuing from the femoral vein of the donor limb was therefore the same as that of the blood entering the femoral artery of the perfused limb.

*Assessment of œdema.* During the perfusion a constant watch was kept for œdema; appearance, measurement and pitting were the main criteria at this stage.

Immediately after death a careful examination was made for any macroscopic evidence of œdema. Tissues from various sites, in particular from below the knees, were then fixed in Zenker's fluid for subsequent histological examination. Further tissue samples, usually 1 to 2 g. in weight, were removed carefully with a razor without displacing any significant amount of fluid, and dropped into dry-weighed bottles fitted with airtight screw-caps. The bottles were reweighed together with their contents. The tissues were then dried to constant weight in a hot air oven at a temperature of 160° C., and their water content was calculated.

Of the various methods of assessing œdema formation, macroscopic and microscopic examination of tissues were only useful in detecting moderate to severe degrees of œdema. The gravimetric method, shown by Heller and Smirk (1932) to be a reliable procedure, provided a very delicate index of œdema in the present study. This is shown by experiments in which œdema was artificially induced by such methods as saline infusions, venous congestion of a limb, or plasmapheresis. Table I shows the close correlation that was found to exist between water content, macroscopic and microscopic appearances of the tissue, and the presence or absence of any of the above factors known to cause œdema.

Table I

*Relationship between tissue hydration, visible œdema and œdema factors*

| Percentage of water in muscles | 73-78 | 78-80 | 80-88 |
|---|---|---|---|
| Macroscopic œdema . . . | none | none | present |
| Histological œdema . . . | none | slight | gross |
| Œdema factors . . . | none | moderate | severe |

The range of values for water content in over 150 biopsies on normal limb muscles of dogs was found to be from 73 to 78 per cent. Values higher than 78 per cent. were found only when factors known to cause œdema were acting on the animal; this figure was therefore fixed as the threshold of œdema in the limb muscles of the dog.

When any "œdema factor" had been operating to a moderate degree, histological œdema was often, though not always, demonstrable by planimetry; the amount of water in the muscles was invariably found to be more than 78 per cent. When any "œdema factor" had been operating to a severe degree, œdema was clearly visible to the naked eye on dissection of the part, and often demonstrable by pitting; histological œdema was gross, and the water content of the limb muscles was more than 80 per cent.

Fig. 2 shows the calf muscles of a dog in which œdema had been produced by plasmapheresis, contrasted with a normal control. In the œdematous muscle (A) the water content was 80 per cent. and the œdema is represented by large interfascicular spaces. The control muscle (B) contained 76 per cent. of water. The distribution of œdema fluid in (A) is of some interest in that the fluid is present mainly in the interfascicular spaces, and is less conspicuous between the muscle fibres; there is no apparent swelling of the cells. This distribution of œdema fluid was invariably found whenever artificial œdema was produced.

## RESULTS

### *Simple perfusion experiments*

Five standard perfusions without important complicating factors were carried out.

Table II illustrates one such experiment performed on a healthy adult dog weighing 19½ kilos. Throughout the 9 hours of this experiment good synchronism was maintained between the revolutions per minute (*r.p.m.*) of the pump and the animal's pulse rate, which increased progressively as the experiment proceeded, owing to the onset of some degree of shock. The blood pressure of the dog was high at the start of the experiment, a common finding in dogs under ether anæsthesia, but fell towards the end when shock supervened.

TABLE II

*Data from a simple perfusion experiment*

| Time after start of perfusion (hrs.) | Animal's pulse rate | Pump R.P.M. | Venous flow from donor limb (ml./min.) | Arterial pressure in perfused limb (mm. Hg.) | Oxygen saturation of perfusing blood (per cent.) |
|---|---|---|---|---|---|
| 1 | 100 | 100 | 100 | 300 | ... |
| 2 | 160 | 160 | 100 | 300 | ... |
| 3 | 150 | 150 | 80 | 220 | 45 |
| 4 | 168 | 168 | 70 | 280 | 62 |
| 5 | 190 | 180 | 65 | 100 | ... |
| 6 | 190 | 180 | 70 | 95 | 62 |
| 7 | 150 | 140 | 55 | 90 | ... |
| 8 | 200 | 180 | 40 | 70 | ... |
| 9 | 200 | 180 | 35 | 70 | 10 |

| Percentage water in limb muscles after death | | | |
|---|---|---|---|
| Perfused limb | Donor limb | Right forelimb | Left forelimb |
| 77·4 | 77·8 | 76·0 | 77·8 |

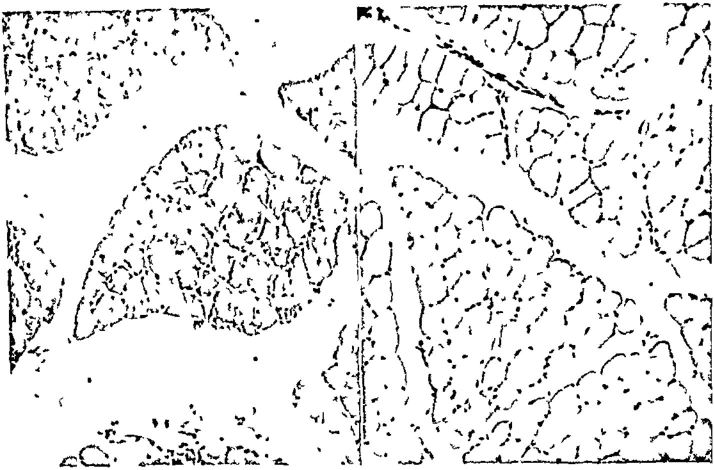

FIG 2

A Œdematous muscle containing 80 per cent water

B Normal muscle containing 76 per cent water

Calf muscle from an œdematous dog compared with the calf muscle from a normal control Hæmatoxylin and eosin ×110

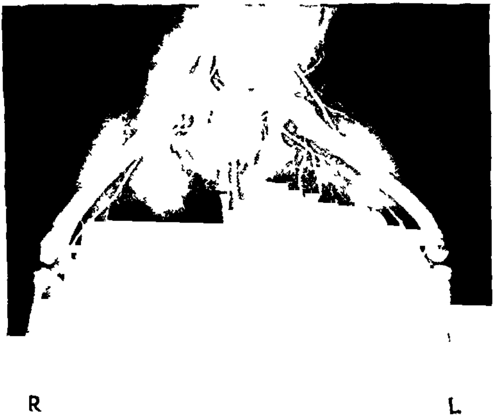

FIG 3—Arteriogram of the hindquarters of a dog The left femoral artery is *occluded by thrombus in the mid thigh region and collaterals have opened up* to bridge the gap ×¼

Similarly the venous flow from the donor limb decreased progressively. The oxygen in the perfusing blood averaged less than 50 per cent. saturation.

At no time during this experiment was there any detectable œdema in the perfused limb or elsewhere. The percentage of water in the muscles after death was essentially the same in all the limbs.

The results of the 5 experiments of this simple type are summarised in table III, from which it is clear that there was no significant difference in hydration between the muscles of the anoxic perfused limbs and those of the normally oxygenated control limbs. All the figures lay within the normal range and the "threshold of œdema" was never exceeded.

Table III

*Results of 5 standard perfusion experiments*

| *Dog* | Duration of perfusion (hrs.) | Oxygen saturation of perfusing blood (per cent.) | | Average percentage water content of limb muscles after death | |
|---|---|---|---|---|---|
| | | Range | Mean | Perfused limb | Control limbs |
| A | 7 | ... | ... | 77 | 78 |
| B | 3 | ... | ... | 77 | 76 |
| C | 9 | 62 to 10 | 45 | 77 | 77 |
| D | 7 | 64 to 32 | 52 | 76 | 77 |
| X | 5 | 58 to 48 | 53 | 76 | 75 |

## *Validity of methods*

*Circulatory condition of the perfused limb.* The arterial pressures of the animals varied during the course of the experiments as shown in table II but in each case the pressure required to perfuse all the blood from the donor limb through the perfused limb was at about the same level as the general arterial pressure of the dog. Table IV gives values for perfusion and forelimb blood pressure in two typical experiments; it shows that the differences between the pressures in

Table IV

*Comparison of brachial arterial pressure and perfusion pressure*

| Time after start of experiment (hrs.) | Blood pressures (mm. Hg.) | | | |
|---|---|---|---|---|
| | *Dog L* | | *Dog M* | |
| | Forelimb | Perfused limb | Forelimb | Perfused limb |
| 1 | 165 | 160 | 135 | 130 |
| 2 | 165 | 150 | 130 | 150 |
| 3 | 160 | 145 | 100 | 115 |
| 4 | 160 | 135 | 90 | 95 |
| 5 | 140 | 125 | 90 | 90 |

the limbs are no more than could be accounted for by normal variations in pressure between the fore- and hind-limbs and by the experimental error of the methods of measurement used.

Pulse pressures, determined in a few experiments, were also found to be of the same order in the perfused limb and in the control limbs. This observed similarity of mean arterial pressures and of pulse pressures shows that the present technique produced no material vasomotor changes in the vascular bed of the perfused limb as compared with the control limbs.

In experiments where venous pressures were recorded it was found that the pressure in the perfused limb was usually a little higher than in the control limbs. The highest venous pressure in the simple perfusion experiments was, however, never greater than 15 cm. saline, which is lower than the venous pressure at which accumulation of fluid occurs in the tissues (Krogh *et al.*, 1932). Table V shows the average venous pressures in 8 representative experiments from the present series.

TABLE V

*Comparison of venous pressures in perfused and control limbs*

| Dog | Venous pressures (cm. saline) | | |
|---|---|---|---|
| | Perfused limb (femoral vein) | Donor limb (femoral vein) | Forelimb (axillary vein) |
| D | 8 | 2 | 1 |
| E | 5 | 1 | 7 |
| F | 10 | 9 | 10 |
| G | 7 | 7 | 8 |
| H | 12 | 10 | 8 |
| I | 10 | ... | 14 |
| J | 15 | ... | 12 |
| K | 11 | ... | 6 |

Variations of the order shown in this table are small. The slight elevation of pressure frequently occurring in the perfused limb might, however, have required separate analysis as a possible factor in increasing capillary transudation if in fact such transudation had increased in any of the experimental animals. This was not the case and the variations are regarded, therefore, as being of no importance in this investigation.

*Capillary permeability in the perfused limb.* Attempts were made in the present study to assess any increase in capillary permeability by means of hæmatocrit and plasma protein estimations. Sometimes comparisons were made between venous blood of the control limbs and blood from the femoral vein of the perfused limb ; at other times values were determined for the arterial and venous blood of the control limbs and compared with the entering and returning blood in the perfused limb. These investigations were carried out to obtain information on (*a*) the transfer of fluid from the blood to the tissues and (*b*) the transfer of protein from the blood to the tissues.

(a) *Fluid transfer.* Hæmatocrit and plasma protein estimations were found to be of no value in assessing fluid transudation through the capillary wall. This was to be expected, however, since a rate of transudation of fluid of a degree likely to be encountered in these experiments would involve insignificant changes in the hæmatocrit and plasma protein levels. The average duration of an experiment was 6 hours and the limb blood flow in a 10 kg. dog was about 50 ml. per minute. The accumulation of 100 ml. of œdema fluid in a limb, though enough to produce overt œdema, would involve differences of only about 0·2 per cent. between arterial and venous hæmatocrits and of about 0·05 g. per 100 ml. between arterial and venous plasma protein levels. These figures are within the limits of the experimental error of the methods of estimation used.

(b) *Protein transfer.* Plasma protein studies gave no evidence of loss of protein from the blood passing through the perfused limb. The variations in plasma protein levels which occurred were not significant. It is concluded, therefore, that the degree of anoxia maintained in the present experiments did not increase recognisably the permeability of the perfused capillaries to protein.

*Effect of perfusion technique on capillary permeability.* That the perfusion technique did not materially reduce the permeability of the capillaries in the perfused limbs was shown by two experiments in which the perfusing blood was diluted by the continuous addition of large amounts of saline. The diluted blood circulated through the capillaries of the perfused limb and produced gross œdema in this limb ; the muscle water in one experiment was 88 per cent. A lesser degree of generalised œdema occurred at the same time in these experiments as a result of the return of the diluted perfused blood to the general circulation. Here the degree of hydræmia was less than in the perfused limb because the diluted perfused blood mixed with the rest of the blood of the animal.

*Degree of anoxia.* Before assessing the results of the present investigation it is important to appreciate the degree of anoxia achieved in the perfused limb. It will be clear that the donor limb received blood at all times at the high oxygen tension of arterial blood (about 90 mm. Hg.) while the perfused limb was supplied at the much lower oxygen tension of venous blood (about 40 mm. Hg.). Oxygen, therefore, was less readily available to the perfused limb besides being present in the blood in much smaller amounts. A comparison of oxygen saturation and oxygen uptake in the perfused and donor limbs based on figures from 7 representative experiments is made in table VI.

This table shows that the donor limb (and this also applies to the fore limbs) received at all times blood almost fully saturated with oxygen, and extracted from it about 5½ volumes of oxygen per 100 volumes of blood. The perfused limb, on the other hand, received blood only half saturated with oxygen and extracted from it on an average only 1 volume of oxygen per 100 volumes of blood.

TABLE VI

*Relative oxygen supply and uptake in perfused and donor limbs*

| | Average oxygen saturation (per cent.) | | Average oxygen uptake (vols. per cent.) | |
|---|---|---|---|---|
| | Range | Mean | Range | Mean |
| Blood to donor limb . | 87-98 | 93 | 3-8 | 5½ |
| Blood to perfused limb . | 30-68 | 52 | ½-2 | 1 |

In the present investigation the anoxia was obtained by perfusion of venous blood which has, in addition to oxygen lack, various other metabolic differences from arterial blood. These other differences which are often quoted as contributory factors in the production of increased capillary permeability, are evidently irrelevant in the present connection since no œdema was produced. Moreover when clinical œdema is ascribed to capillary anoxia, the implication is that this is due to increased venosity of the circulating blood.

*Collateral circulation.* Despite the apparently adequate supply of blood from the pump to the perfused limb, it is necessary to consider whether this limb might also have been receiving an oxygen supply from collateral vessels. Angiography shows in fact that a collateral circulation can be opened up very quickly. This point is illustrated in fig. 3 which is an arteriogram of the pelvis and hindlegs in a dog. The right femoral artery in this animal was intact and it is apparent that the tissues below the knee are supplied exclusively by the femoral artery. The left femoral artery had been occluded at about mid-thigh for about twelve hours and within this short time, a collateral circulation had opened up, bridging the gap in the main vessel and supplying blood to the lower part of the leg. The question arises whether the operation on the femoral artery and the perfusion of the limb allows any such collateral circulation to be established during the course of an experiment. *A priori* considerations make it highly improbable that such collateral circulation could play any important part in these experiments. The work of Richards (personal communication) has shown that the contribution from collateral vessels to the main arterial tree is negligible until the mean pressure of arterial blood in the main vessels falls to a low level : his results do not suggest that any factors other than purely mechanical ones were operative. In the present investigation the mean arterial pressure in the perfused artery was always very close to the mean general arterial pressure of the animal. The capillaries in the perfused limb below the knee can therefore justifiably be regarded as supplied only by the perfusing blood.

Direct evidence on this question is provided by a perfusion experiment in which all possibility of collateral circulation either in the perfused or in the donor limb was eliminated completely. This was accomplished, after section of the skin of these limbs just below the inguinal ligament, by very tight ligation of the entire leg in three tissue bundles, excluding only the sciatic nerve, the femoral nerve, and the femoral vessels. The results of this experiment differed in no way from the results of the simple circulation experiments without mass ligature: the water content of the muscles in the perfused limb showed no significant difference from that in the control limbs (76 per cent. and 77 per cent. respectively). Thus, though the unlikely possibility of slight collateral circulation in the 5 standard experiments cannot be entirely excluded, the provision of oxygen by such a circulation was not the explanation of the absence of œdema in the perfused limb in those experiments.

*Exercise.* A further possibility to be considered is that the effects of anoxia might not be evident in a limb at rest but might be made manifest by exercise. This requires consideration particularly since Lazarus-Barlow (1894) has shown that exercise aggravates post-ischæmic œdema in dogs. An experiment was designed, therefore, to determine whether or not exercise would have such an effect:—

A standard perfusion experiment was set up and both hindlimbs of the dog were subjected to continuous faradic stimulation mainly applied directly to the muscles below the knee, but with extra stimulation applied from time to time to the sciatic and femoral nerves. This simulated exercise was maintained for a period of 4 hours. No œdema was produced in the experiment; the degree of hydration in the muscles of the anoxic and the normally oxygenated limbs remained quite normal (76 per cent. in both).

*Latent effects.* Heimberger (1926-27) demonstrated that when human capillaries are subjected to ischæmia, mechanical irritation does not produce local capillary reactions until normal blood-flow through them has been restored. Such a possibility must be considered in the present study. Three experiments were carried out to determine whether capillary reactions might be latent during the period of anoxic perfusion, becoming recognisable only when a normal circulation was restored.

Standard perfusion experiments were established and maintained for about 4 hours. All cannulæ were then removed and the incisions in the vessels sutured with fine silk and atraumatic needles, so that the normal circulation to the hindlimbs was restored. The skin wounds were closed and the dogs were left to recover from the anæsthetic. No further heparin was given in order to avoid oozing of blood from the wound sites. Strict aseptic precautions were observed throughout these experiments.

In two experiments the animals survived for only about 4 hours; the deaths appeared to be the result of shock. The femoral vessels were patent in both legs. In neither of the dogs was there any œdem of the perfused or the control limbs. In the first experiment t muscles of the perfused limb contained 76 per cent. of water and th control limb 77 per cent.; in the second experiment they bot contained 77 per cent.

In the third experiment the animal recovered completely. Pulsation in the vessels below the knee was undiminished 8 hours aft suture. Occlusive thrombosis subsequently developed in the dono vein and the perfused artery as illustrated in fig. 3 and the obstructio of the artery appeared to be fully compensated by the opening up o collaterals. Despite this late thrombosis, there was no œdema o either the perfused or the control limbs; they showed a muscle wate content of 76 and 75 per cent. respectively.

These survival experiments give no evidence of latent damage t capillary endothelium in the anoxic limb as judged by subsequen changes of tissue hydration.

### *The effect of anoxia on œdema produced by other methods*

The foregoing standard experiments show that local capillar anoxia of a severe degree fails to produce œdema; it remains to b considered whether it can aggravate œdema caused by other agents.

*The œdema of hydræmia.* Four experiments were performed i which generalised œdema was produced by the administration o moderate and large quantities of saline intravenously into the general circulation of the animal during a perfusion experiment. Table VII shows that in these experiments the extent of the œdema was the same in the perfused and control limbs. Anoxia had not aggravated the œdema of hydræmia.

TABLE VII

*Œdema produced by different degrees of hydræmia. Comparison of muscle hydration in perfused and control limbs*

| Dog | Duration of experiment (hrs.) | Total saline given (ml./kg.) | Œdema | | |
|---|---|---|---|---|---|
| | | | Generalised (*macroscopic*) | Muscle water content (per cent.) | |
| | | | | Perfused limb | Control limb |
| N | 4 | 50 | — | 78 | 78 |
| E | 3 | 75 | ± | 78 | 79 |
| O | 2 | 150 | ± | 79 | 78 |
| P | 5 | 650 | + | 78 | 79 |

*The œdema of plasmapheresis.* Acute hypoproteinæmia was induced in one dog by several plasmaphereses carried out during the 8 hours of an otherwise uncomplicated perfusion experiment. The final plasma protein level attained was 1·7 g. per cent. Ascites and generalised œdema developed. The perfused and control limbs each showed an average muscle water content of 80 per cent., and there was thus no evidence that the anoxia had aggravated the effect of low plasma protein.

*The œdema of venous congestion.* Four experiments were designed to determine whether or not anoxia would increase the rate of development of œdema due to raised venous pressure.

In these experiments the perfusion was started in the usual manner and inflatable cuffs were applied to the perfused limb and to one or more of the control limbs. The venous pressures in all these limbs were recorded continuously. They were increased by inflation of the cuffs and maintained constant by adjustment of the cuff pressure as required. The cuffs were kept inflated for periods of 4 to 5 hours and were removed a minute or two before the circulation was stopped and the animal killed. The cuffs were removed before death to allow the excess of blood in the congested limbs to escape into the general circulation. (Krogh *et al.* have shown that this escape is practically completed within 45 seconds of the release of the cuffs.)

Information from other experiments was also analysed to determine the degree of hydration in both oxygenated and anoxic limbs at venous pressures within the normal range.

The results of these investigations are summarised in table VIII which shows (*a*) that œdema developed in both the control and perfused limbs in proportion to the increase of venous pressure above 23 cm. saline, and (*b*) that the degree of the œdema was not significantly different in the perfused limb and the control limb at the same venous pressures. There is thus no evidence that anoxia has any effect on the œdema of venous congestion.

TABLE VIII

*Influence of venous pressure on œdema production. Comparison of muscle hydration in perfused and control limbs*

| Venous pressures (cm saline) | Muscle water content | |
|---|---|---|
| | Perfused limb (per cent ) | Control limb (per cent ) |
| 15 or less | 76* | 77* |
| 23 | ... | 77+ |
| 29 | 79 | 78 |
| 30 | 78 | 79 |
| 33 | 79 | 78 |
| 36 | 80 | 80 |
| 48 | ... | 81 |

* Average figures from three experiments

*Artificially produced local œdema.* Experiments were carried out to determine whether anoxia would have any effect on (*a*) the rate of re-absorption of fluid injected locally, and (*b*) the development and re-absorption of local hypersensitivity œdema.

(a) *The absorption of local depots of fluid*:—A standard perfusion experiment was established.

*Physiological saline* was then injected subcutaneously in 3 ml. doses into the loose tissues of the dorsal surfaces of both hind feet of the dog to produce local fluid swellings. There was no detectable difference in the rate of disappearance of the saline from the anoxic perfused limb and the normally oxygenated donor limb; both swellings had almost disappeared within the course of an hour.

*Normal dog serum* injected intradermally in 0·1 ml. doses produced small and sharply defined blebs. These disappeared at the same rate in both the perfused limb and the donor limb.

From these experiments it appears that re-absorption of fluid with or without a protein component is unaffected by capillary anoxia.

(b) *The local œdema of serum sensitivity.* An anæsthetised 10 kg. dog was sensitised to horse serum by means of an intravenous injection of 25 ml. of the serum. A month later a standard perfusion was established in the animal and the local reactions to 0·1 ml. of horse serum intradermally in both hind limbs were studied. These injections of horse serum produced large wheals, about twelve times the size of control injections of 0·1 ml. of normal dog serum. No difference was observed in the rate of development, the maximum size, or the rate of disappearance of the wheals in the anoxic perfused limb and the normally oxygenated donor limb. Anoxia apparently failed, therefore, to increase the permeability of already damaged capillaries or to retard reabsorption of the local œdema fluid.

### *Influence of* (*a*) *drugs and* (*b*) *lymphatic drainage*

The foregoing experiments show that severe capillary anoxia had no detectable influence on the water content of tissues. It remains to be shown whether (*a*) increased filtration from capillaries was prevented by the general condition of the animals studied, or (*b*) increased filtration was masked by increased lymphatic drainage in the perfused limb.

(*a*) The general condition of the experimental animals might, in theory, have prevented the development of increased capillary permeability in the anoxic perfused limbs. For example, histamine release might be partially inhibited by a high blood heparin level (Rocha e Silva *et al.*, 1947). Against such a possibility must be set the fact that most of the fundamental types of œdema have been produced under the present experimental conditions. Control experiments were carried out to determine whether the experimental conditions had any partial inhibitory effect on hypersensitivity œdema; this type of œdema is evidently due to increased capillary permeability and therefore of special interest in the present context. From these experiments it was found that the wheals of intradermal horse serum in a sensitised dog developed at the same rate, reached the same size and were absorbed at the same rate, (*a*) before atropine, Nembutal and heparin were given, (*b*) after the administration of these drugs, as in the preliminary medication in the perfusion experiments, and (*c*) during a standard perfusion experiment. It appears, therefore, that the experimental conditions had no inhibitory effect on the production of œdema.

(*b*) Increased lymphatic drainage might, in theory, have been responsible for failure to develop œdema in the perfused limb in the standard experiments. The complete symmetry of the œdema produced by saline infusion, plasmapheresis and local injection strongly suggests that this was not the case. In these experiments it is extremely unlikely that the lymph-flow from the limbs would have increased in such precise unequal proportions as to compensate exactly for possible effects of the anoxia. Direct evidence on the point is provided by the experiment previously described, in which collateral circulation was eliminated. Lymphatic drainage in this animal would be reduced almost to nothing, being confined to those few channels, in close juxtaposition to the femoral vessels and the femoral and sciatic nerves, which were not included in the mass ligatures. Such restriction of lymph flow produced results identical with those in the standard perfusion experiments. In a similar mass ligature experiment in which œdema was produced by saline infusion (dog E), there was no significant difference in the degree of œdema which developed in the perfused and control limbs. The unimportant role of the lymphatics in the present context is not unexpected, since Drinker and Yoffey (1941) have shown that lymphatic drainage in mammals is a sluggish process even in the presence of œdema, unless active measures to promote flow, such as massage, are used. Moreover, Cameron *et al.* (1947-48) have produced the œdema of capillary damage in animals by exposing them to lewisite; they observed that while the lymph-flow in these animals was increased, the production of œdema fluid was much faster than its removal.

## DISCUSSION

The literature contains studies of the effects of all types of anoxia. The methods by which these effects have been investigated include (*a*) clinical observation; (*b*) experiments on man, using plethysmograph, and plasma protein and hæmatocrit techniques; and (*c*) experiments on animals in which more direct methods of assessing fluid transfer from the capillaries are used, such as the measurement of lymph flow or capillary microscopy.

In the present study the effect of local capillary anoxia in dogs has been assessed principally by a gravimetric technique. The anoxia obtained was of the anoxic type and of a degree greater than that encountered in pathological states asociated with œdema. Harrop (1919), for example, found that the percentage oxygen saturations in the arterial blood of 9 cases of decompensated heart disease, lay between 81·4 and 97·7, which is considerably greater than the percentage saturation of oxygen in the perfused blood in the present experiments. The finding that perfusion with extremely anoxic blood failed to cause œdema, to accentuate œdema produced by other methods, or to delay the re-absorption of œdema fluid, indicates the need for a critical examination of the literature on the effects of anoxia on capillary permeability and the production of œdema. As far as possible the literature is classified according to the type of anoxia mainly concerned.

### *General anoxia*

Most studies on the relationship between anoxia and the permeability of capillaries are based on the effects of general anoxia. At the outset it must be emphasised that such investigations cannot prove the existence of a direct action of oxygen lack on capillary endothelium, since the possibility that the anoxia operates through the central nervous system or by some other central mechanism, cannot be excluded.

*Acute anoxic anoxia* may be obtained by exposing men or animals to a low partial pressure of oxygen: oxygen is present in the blood in small amounts and at low tension.

McMichael and Morris (1936) found that in human subjects, swelling of an arm congested by a cuff occurred at a uniform rate. This rate was not increased when the subjects became anoxic by breathing air poor in oxygen; the degree of anoxia achieved was equivalent to an arterial saturation of about 65 per cent. Henry *et al.* (1947) in similar limb congestion experiments detected no increase in capillary permeability to protein in states of mild anoxic anoxia, a finding which supported earlier work (Henry *et al.*, 1946); when, however, the oxygen saturation in venous blood draining from the limb fell to 15 to 25 per cent., a significant increase in capillary permeability to protein was observed.

Hopps and Lewis (1947) were unable to demonstrate in anoxic guinea pigs any increase in capillary permeability to antibodies, plasma proteins or dyes. The animals were exposed to oxygen tensions corresponding to an altitude of 30,000 ft., which is about the minimal lethal altitude for human beings. Calvin (1941) on the other hand, concluded from an investigation into the effects of *asphyxia* on plasma volume, red cell volume, and plasma protein concentration in animals that from moderate asphyxia to the point of cessation of respiratory movements, plasma volume progressively decreased. This suggested an increase in capillary filtration as a result of the direct action of very severe anoxia on the endothelium. Swann and Brucer (1949) have, however, shown that the increases in plasma protein levels in dogs subjected to fatal anoxia occur only when the animal is under general anæsthesia: Calvin's interpretation that the changes in plasma protein levels indicate capillary leakage of fluid is not accepted by these workers.

In anoxic dogs, Maurer (1940-41) observed an increase in flow of cervical lymph and an increased passage of protein from the blood capillaries to the lymph. In this study, the results of which were later confirmed by further work on dogs breathing air containing carbon monoxide (Maurer, 1941), he noted a maximum flow of lymph when the arterial oxygen saturation of the dogs fell to 52·5 per cent. This worker observed general circulatory changes in the anoxic dogs but believed that these were insufficient to account for the increased lymph-flow ; the evidence for this opinion is not conclusive.

Cameron and De (1949) have shown that in rabbits severe respiratory obstruction, pushed to the extremes of asphyxia and maintained for several minutes, failed to give lung œdema. Such an absence of œdema does not prove that capillary permeability in the lung is quite unchanged in anoxic animals since Warren and Drinker (1942) demonstrated an increase in lymph flow from the lungs of anoxic dogs. However, the mode of production of œdema in the lungs is probably essentially different from that in other parts of the body. In the present study pulmonary œdema was not observed in the animals in which generalised œdema had been produced by intravenous saline infusions or plasmapheresis.

Various observations made on men and animals subjected to low barometric pressures show that severe anoxic anoxia can occur without associated œdema. Many accounts of experiments on men and animals subjected to low oxygen tensions and a number of autopsy reports on patients, principally airmen dying of anoxia, are to be found, in which evidence of anoxia is convincing but neither œdema nor effusions are ever mentioned as findings (Lewis *et al.*, 1942 ; Van Liere and Stickney, 1943 ; Ward and Olson, 1943 ; Gibbs *et al.*, 1943 ; White, 1947 ; Lewis and Haymaker, 1948). Swann and Collings (1943) actually produced dehydration of the tissues of rats by subjecting them to low barometric pressures. Some of these latter observations may, however, be unimportant in the present context for the following reasons. Firstly, at low barometric pressures, insensible water loss from the body is presumably increased. Secondly, unless extra water were available, the fluid in the circulatory system would be insufficient to cause generalised œdema which could be recognised macroscopically.

*Chronic anoxic anoxia* occurs clinically in cases of congenital heart disease in which there is a shunt between the right and left sides of the heart. Although the anoxia in these patients is to some extent ameliorated by the accompanying polycythemia, their arterial blood has a low oxygen saturation : the range in a series of cases of Fallot's tetralogy is given by Taussig (1947) as 10 to 88 per cent. Despite this chronic anoxia, œdema is very rarely observed in these cases (Fahr and Ershler, 1941).

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### *Local anoxia*

One of the classical investigations on the effect of anoxia on capillary permeability was made by Landis (1927-28). He produced local capillary anoxia in the mesentery of frogs by stopping the blood flow for a few minutes and irrigating the surface of the mesentery with deoxygenated Ringer solution. He found that there was an increased transudation of fluid from the capillary he was investigating, as compared with that from the control capillaries exposed to oxygenated Ringer solution. He noted some lack of uniformity in his results and attributed this to the proximity to the capillary he was observing of other capillaries containing freely circulating blood. This suggested that the degree of anoxia required to produce increased fluid transudation was severe, in fact so severe that in 1934 (p. 462) he stated " . . . it is still doubtful whether the oxygen tension in functioning tissues ever becomes low enough to be of importance."

An objection to applying the results of Saslow's and of Landis's experiments to mammalian pathology lies in important differences which exist between the circulatory dynamics of frogs and mammals (Isayama, 1924-25; Florey, 1926; Conklin, 1930*a*). It is also well known that the capillaries of frogs have normally a much greater permeability to protein than have those of mammals (Conklin, 1930*b*; Rous and Smith, 1931).

*Ischæmia.* Total vascular occlusion for some hours in experimental animals has been shown to cause œdema after the circulation is re-established (Lazarus-Barlow; Pochin, 1939-42). This effect of local ischæmia is often put forward as evidence that anoxia increases capillary permeability though it is scarcely necessary to point out that in ischæmia the capillaries are deprived of all nourishment and not simply of oxygen. It is impossible to say from such experiments which particular effect of the ischæmia causes the œdema.

### *Conclusions from the literature*

It is clear from the published work that acute anoxia increases capillary permeability only when the anoxia is extremely severe. Acute anoxia of this degree is to be expected only in an animal on the point of death. There is no evidence that a milder degree of anoxia persisting for a long period has the effect of increasing capillary permeability and producing œdema.

## SUMMARY

In order to obtain direct evidence of the effects of local anoxia on capillary permeability and the production of œdema, experiments were performed under controlled conditions on dogs in which the femoral artery of one hindlimb was perfused with venous blood from the femoral vein of the opposite hindlimb. The average oxygen

saturation of the perfusing blood was in the region of 50 per cent. and the duration of the perfusion was usually 6 to 7 hours.

In one group of experiments uncomplicated perfusions were carried out; œdema did not develop in the anoxic perfused limb. In a second group of experiments various modifications of the simple perfusion technique were made, and these confirmed the validity of *this method of investigating œdema. In the remaining experiments,* the perfusion method was used in dogs in which œdema had been produced by saline infusion, plasmapheresis, venous congestion or local injection; anoxia did not aggravate the œdema in the perfused limb nor reduce its rate of re-absorption.

The evidence from these experiments and from a critical analysis of the literature suggests that capillary anoxia plays no part in the production of œdema.

I am indebted to Professor H. L. Sheehan for his guidance and criticism throughout this investigation, to Professor R. A. Gregory for his advice on physiological technique, and to Dr Helen T. Morgan for her assistance during experiments.

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# MYONEURAL JUNCTIONS AND TOXIC AGENTS

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(Plates XXXVII-XLI)

The myoneural junction which forms the vital link between nerve and muscle is being studied intensively by physiologists and almost entirely neglected by pathologists. This neglect is surprising because an increasing number of toxic agents are being shown to produce their effects at this site. Investigations in the field of myoneural pathology have been directed chiefly to the study of the degenerative changes which follow section of the motor nerve, and the fact that similar changes follow the destruction of the neurone in poliomyelitis (Chor, 1933; Dublín *et al.*, 1944) has been accepted as a reasonable and expected sequel. Interest in the human myopathies has usually been confined to the muscle itself, but Noël and Pommé (1932) reported primary changes in the motor end-plates. On the other hand, Falin and Kanarejkin (1941) described changes which they considered to be secondary to the atrophic process in the muscle. The lesions of the myoneural junctions which have been reported in beriberi will be considered later. This short summary disposes of all that is reasonably definite in the pathology of the myoneural junctions and leaves for consideration the much more obscure problem of the effect of drugs and bacterial toxins, grouped generally as toxic agents, on the microscopic anatomy of the myoneural junction.

In a review of the mode of action of a number of toxic agents, Dale (1948) has pointed out that although curare, botulinus toxin and tetanus toxin all produce their effects at the myoneural junction, the precise mode of action is different in each case. The physiological differences are definite but the claims that morphological changes can be demonstrated are conflicting and inconclusive. In the present work the compound para-nitrophenyl diethyl thiophosphate, to which the symbol E.605 is given, has been used to produce functional disturbances at the myoneural junctions and to provide material for histological study. The symptoms of E.605 poisoning, as reported earlier (Denz, 1951), include muscular fibrillation and weakness. E.605 has the power of inactivating both true and pseudo-cholinesterase and probably produces its effects *in vivo* in this way (DuBois *et al.*, 1949). The prevailing view of the transmission of nervous impulses from motor nerve ending to muscle fibre is that a minute amount of acetylcholine is liberated at the end-plate. This acetylcholine is almost immediately hydrolysed by cholinesterase, but it persists long enough to cause an excitatory depolarisation at the surface of the motor end-plate and the impulse is then electrically propagated in the muscle fibre. In E.605 poisoning, the cholinesterase is inactivated and therefore acetylcholine accumulates and persists at the myoneural junction. Thus a mechanism depending on the sudden production and rapid disappearance of acetylcholine for its initiation breaks down if acetylcholine persists unchanged. The fibrillation and muscular weakness probably result from this disturbance in the transmission of nervous impulses.

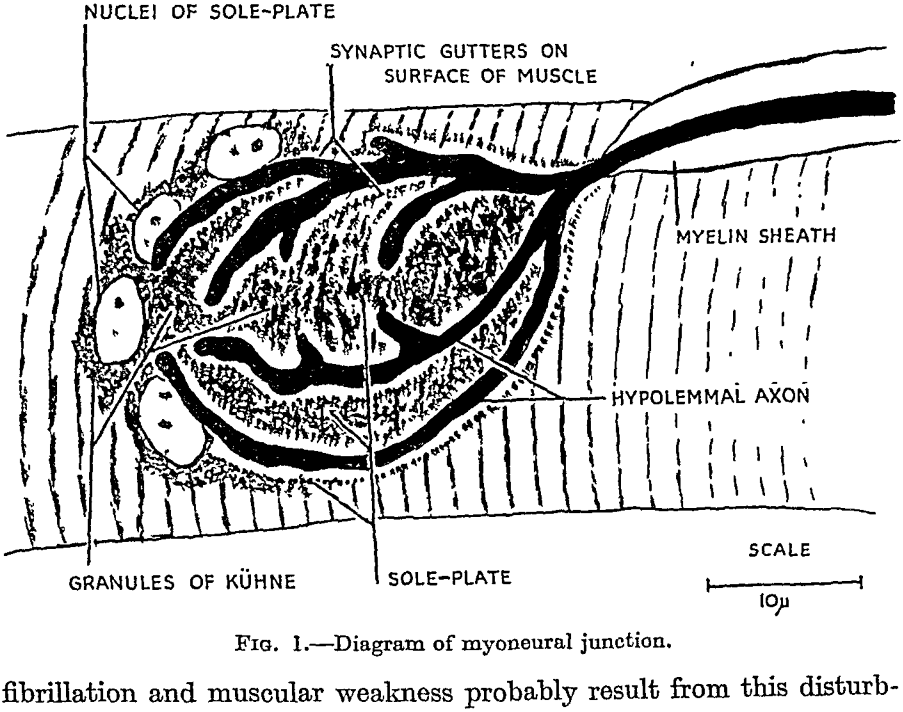

FIG. 1.—Diagram of myoneural junction.

Since motor end-plates were first described by von Doyere in 1840, their structure has been the subject of considerable controversy. Stöhr (1928), Boeke (1932) and Hinsey (1934) have given full accounts of the earlier work, and more recently Tello (1944) and Couteaux (1947) have again attempted the elucidation of the finer structure. The terms myoneural junction and motor end-plate will be used synonymously in this paper. A labelled diagram (fig. 1) gives some idea of the modern views of the structure of this organ and of the components to which reference will be made in the text. The myelin sheath of the nerve fibre ends just before the fibre ramifies to form the hypolemmal axon, which has been shown by Couteaux to be in shallow gutters on the surface of the muscle fibril. The muscular component

MYONEURAL JUNCTIONS AND TOXIC AGENTS

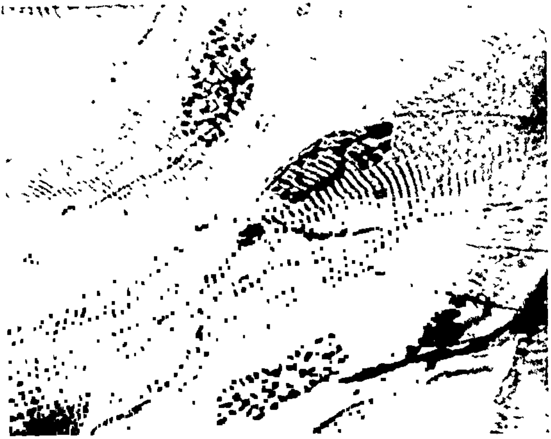

FIG. 2.—Three muscle end-plates of average size, from a rat poisoned acutely with E.605. Gold chloride. ×750.

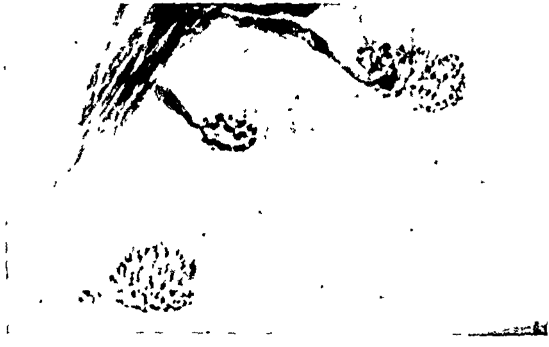

FIG. 3.—Three end-plates, one slightly out of focus. Two are of average size and one is very small (about 12 μ in length). From a rat poisoned acutely with E.605. Gold chloride. ×750.

MYONEURAL JUNCTIONS AND TOXIC AGENTS

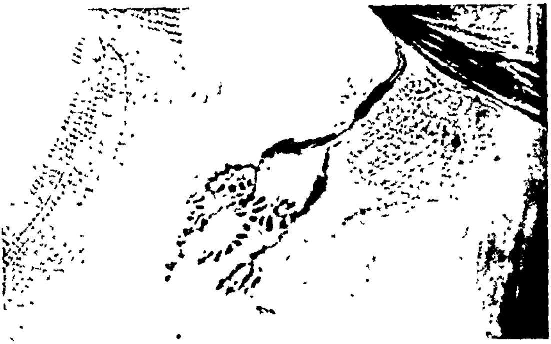

FIG. 4.—Large end-plate for comparison with fig. 3. From a rat poisoned acutely with E.605 Gold chloride. × 750.

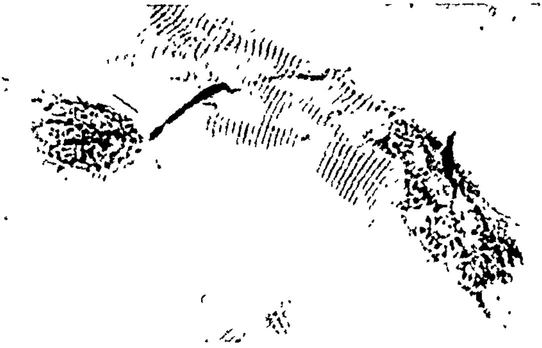

FIG. 5—One end-plate of average size and one large end-plate (about 50 $\mu$ in length). Granules of Kuhne prominent, with clear areas around fronds of hypolemmal axon From a rat poisoned acutely with E.605. *Gold chloride.* × 750

MYONEURAL JUNCTIONS AND TOXIC AGENTS

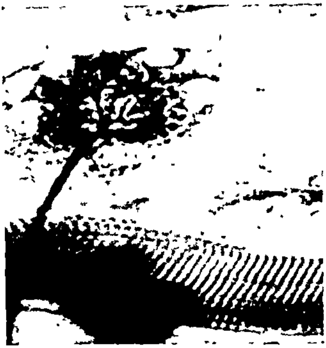

FIG. 6.—End-plate showing well-developed granularity of the sole-plate. The granules of Kuhne outline the nuclei of the sole plate, which appear as clear oval spaces. From a rat poisoned acutely with E 605. Gold chloride. ×750

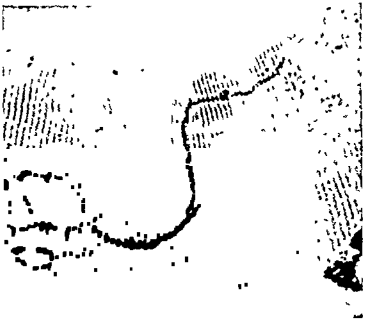

FIG 7.—End-plate with agranular sole-plate. For comparison with figs. 5 and 6. From a rat poisoned acutely with E 605. Gold chloride. ×750.

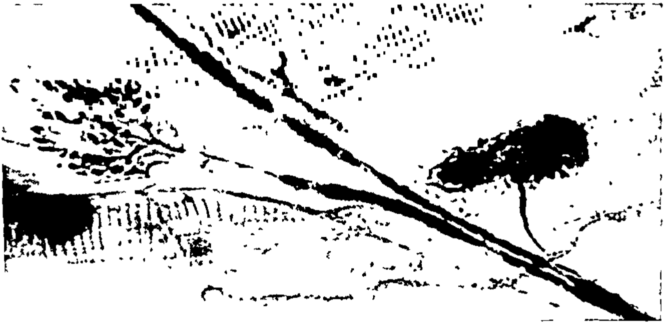

FIG. 8—To illustrate the variation found in adjacent end-plates. One is of Carey's expanded type, the other of the retracted, strongly chrysophilic type. From a rat poisoned acutely with E.605. Gold chloride. ×750.

MYONEURAL JUNCTIONS AND TOXIC AGENTS

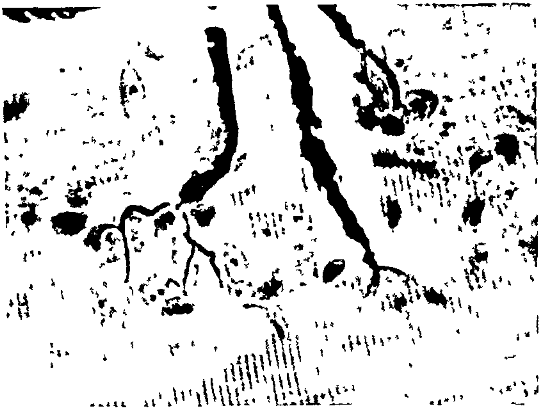

FIG 9—Three myoneural junctions one of which shows some of the hypolemmal axons sharply in focus The collection of nuclei around the myoneural junctions is well shown Silver method—Bielschowsky Gross, counterstained with hæmatoxylin ×750

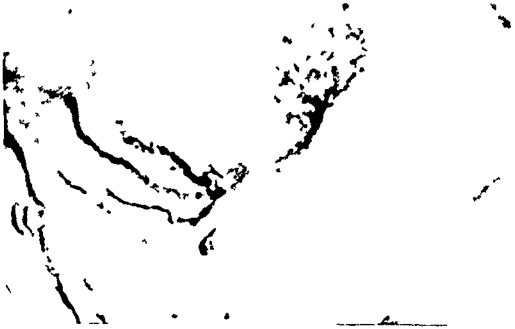

FIG 10—The first subdivisions of the hypolemmal axon are sharply in focus, but the rest lying in a deeper plane are much less well defined From an animal poisoned acutely with E 605 Methylene blue ×750

of the myoneural junction is the sole-plate. In gold preparations this is frequently studded with granules, the granules of Kühne, which outline the pale areas occupied by the nuclei of the sole-plate (fig. 6) and the margins of the gutters containing the neural ramifications (fig. 5). The muscle striations continue across the sole-plate (figs. 2 and 7), but are less well defined and are frequently obscured by the granules of Kühne (fig. 5).

The three different methods used in this work—the gold, silver and methylene blue methods—tend to show up different components of the end-plates, as Wilkinson (1929) has stated. The diagram (fig. 1) is drawn more from the appearance with gold preparations, and does not indicate the fineness of the neural ramifications obtained with the silver method (fig. 9). Again, the granules of Kühne are frequently present in gold preparations, but only rarely in methylene blue preparations (fig. 13) and never in silver preparations, although Carey (1944*a*) has implied that their counterpart in silver preparations is that capricious and controversial structure the periterminal network. The problem of comparing the results obtained by the different methods is avoided to some extent in this paper by considering separately the results obtained with each method in turn.

An outstanding feature of motor end-plates is the great variability of the neurofibrillary network. No end-plate is ever exactly like another, and this individuality has led to encyclopædic accounts such as that of Kühne (1887), who described no less than 300 different types of end-plates. One explanation of this diversity of structure is that these variations reflect changes in physiological state, a concept that has been advanced in particular by Carey and his associates. The present work was designed to see whether lesions could be produced at the myoneural junctions by an anticholinesterase agent and to investigate this relationship between morphology and physiological state of the motor end-plates. It includes a critical review of the contributions of others to this question. The technical problems in the making of microscopical preparations of myoneural junctions are considered in detail, for an appreciation of these problems is crucial in the assessment of work in this field.

## METHODS

The albino rat was used in the majority of the experiments and discussion will be limited to this animal. Some preparations were made from the tissues of mice and rabbits but these do not affect the conclusions reached on material from the rat. Acute E.605 poisoning was induced by subcutaneous injection of solutions of E.605 in ethyl lactate or ethyl alcohol, the dose ranging from 1 to 15 mg. per kilo. Prolonged poisoning with E.605 was produced by daily injections of 1·5 mg. per kilo for periods up to ten days. Symptoms of prolonged poisoning were also produced by feeding on a diet containing 100 parts per million of E.605. The rats were killed by a blow on the head. The muscles examined were the intercostals, the abdominal muscles and the gastrocnemius.

For the gold chloride method the tissue was fixed immediately after death in 20 per cent. citric acid. Small pieces of muscle were treated with gold chloride solution according to Carey's (1942) modification of Löwit's (1875, quoted by Hinsey) original method. Crush preparations of the muscle were found to be most satisfactory.

Two different methods were used for silver preparations. Evans's modification (1949, personal communication) of the Bielschowsky-Gross method was used chiefly. The best results were obtained after fixation for 7 or 8 months in the pyridine-formalin solution. A smaller number of preparations were prepared by Glees' (1946) modification of the silver method.

The methylene blue method used in this work was evolved only after meeting many technical difficulties. The innumerable variations of the methylene blue method which have been published since Ehrlich first used this dye to stain nerve tissue in 1886 emphasise its capricious behaviour. The problem has been somewhat clarified by Schabadasch (1935, 1936), who summarised in two papers the results of twelve years' work. On the basis of Schabadasch's findings a staining solution was designed to stimulate glycolysis by the addition of glucose and of magnesium and phosphate ions. The solution consisted of sodium chloride 0·8 per cent., glucose 0·2 per cent., magnesium bromide 0·02 per cent., methylene blue 0·02 per cent., buffered to *p*H 6 with a phosphate buffer. Methylene blue 2 B.S. (I.C.I.) was found to be the best of a series of six different samples, but no explanation can be given for this finding. Both control rats and those poisoned with E.605 were killed by stunning. The chest wall was immediately exposed and the intercostal muscles infiltrated with the staining solution, which had been warmed to 37° C. The whole animal was then placed in an incubator for 20 minutes, the intercostals being infiltrated twice more with the methylene blue solution. The "blueing" was controlled under a dissecting microscope and small pieces of muscle containing stained end-plates were selected for fixation in ammonium molybdate or ammonium picrate Frozen sections were cut, dehydrated in *n*-butyl alcohol, cleared in xylene and mounted in canada balsam. Satisfactory although not quite uniform staining could be obtained consistently with this method.

## RESULTS

### *Gold chloride method*

The basis of comparison of the end-plates of animals poisoned with E.605 and normal animals was :—(1) The size of the end-plates. (2) The appearance of the hypolemmal axon, *i.e.* the nervous ramifications within the end-plates. (3) Alterations in the granularity of the sole plate—the granules of Kühne. (4) The incidence of gross lesions such as nuclear pyknosis or fragmentation of the end-plates.

The maximum diameter of the end-plates was measured in both experimental and control animals. The mean and standard deviation for 500 consecutive end-plates for the two groups were :—

| | |
|---|---|
| Normal animals . . . | 33·65 $\mu \pm 7{\cdot}231$ |
| Poisoned animals . . . | 33·37 $\mu \pm 7{\cdot}915$ |

More detailed analysis of the figures did not disclose any significant variation from preparation to preparation or from animal to animal. The end-plates of chronically poisoned animals were also of normal dimensions.

The variation in size of end-plates can be illustrated by reference

## MYONEURAL JUNCTIONS AND TOXIC AGENTS

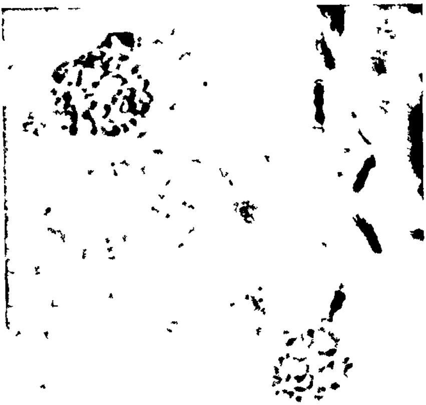

FIG. 11.—Staining chiefly of the hypolemmal axon, the variation in intensity being due to the fact that the sole plate is not a flat structure and only parts of the hypolemmal axon are in sharp focus. Methylene blue. ×750.

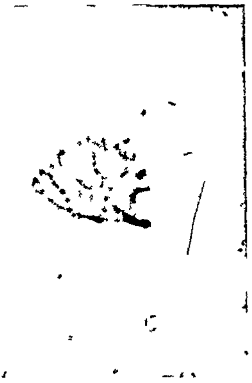

FIG. 12.—The staining here is confined almost entirely to the gutter of the sole plate in which the hypolemmal axon lies. Methylene blue. ×750.

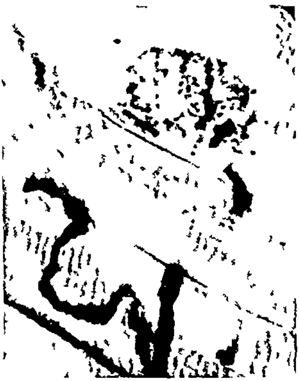

FIG. 13.—Dark staining of hypolemmal axon and somewhat irregular deposition of granules in the sole plate. Methylene blue. ×750.

to the following photographs, all of which have the same magnification. Fig. 2 provides examples of end-plates of average size as stained by the gold method. Fig. 3 shows two end-plates of average size and one very small end-plate which may be contrasted with the large end-plate in fig. 4. Fig. 5 also shows a very large end-plate as well as one of more moderate size. As these are all from poisoned animals it is obvious that E.605 poisoning has not produced a common expansion or retraction of the end-plate, but has left intact the normal variability.

The hypolemmal axon in the gold method is outlined by the fine granules. Considerable variations in granularity are found in both control and experimental animals. A consideration of the technical details of the method suggests that great caution must be used in attributing such variations to physiological or toxic changes. It is customary to make several preparations from a single piece of muscle, usually between five and ten. Some of these may not show any end-plates; others show end-plates stained with varying degrees of precision. From the set of preparations one or more is chosen because of the clarity or elegance of the end-plates and taken as representative of the muscle. In this way each preparation is selected for some property decided on by the experimenter before it is used for assessment of changes attributable to toxic agents. Alterations in the granularity of the hypolemmal axon may arise from differences in the time the tissue is taken after death, in the relation of the end-plates to the surface of the muscle exposed to the gold chloride, in the thickness of the fragment of tissue, in the strength of the gold solution and probably many other factors. In a single preparation some end-plates may be well stained, others overstained, and yet others so lightly stained as to be almost invisible. These variations and the process of selection of preparations for detailed examination make comparison of tissues from control and experimental animals fraught with possible sources of error. No constant change in granularity of the hypolemmal axon has been found in E.605 poisoning. Indeed the experimental and control tissues are indistinguishable.

Alterations in the appearance and number of the granules of Kuhne have been described by Carey (1944*a*) following the administration of curare and quinine, and to a much lesser degree by Guyton and MacDonald (1947) in experimental botulinus poisoning. The granularity of the sole-plate is as variable as the granularity of the hypolemmal axon, and the same criticisms of its validity as a criterion of abnormalities of the muscle end-plates can be applied. Thus figs. 5 and 6 show well-defined granules of Kuhne whereas figs. 4 and 7 are almost without granules. Fig. 8 shows two end-plates on adjacent fibrils, one being of Carey's expanded type showing few granules, the other a retracted type dark with granules. Thus in E.605 poisoning no consistent change could be found in the granules of Kühne in the sole-plate.

Gross changes in the end-plates were sought but not found. No single feature or combination of features was found that could enable the myoneural junctions of E.605-poisoned animals to be distinguished from those of normal animals. All the illustrations are from animals poisoned with E.605. A similar series could be selected from normal material.

### *Silver method*

Clean and satisfactory preparations were obtained with the silver method. The fine ramifications of the hypolemmal axon as displayed with this method (fig. 9) contrast strongly with the coarse fronds shown by the gold method. In most preparations only the hypolemmal axon is seen and there is little to characterise the sole-plate except its nuclei which in fig. 9 have been stained with hæmatoxylin. The method has the disadvantage of sectioned material as compared with crush and teased preparations in that myoneural junctions are so large that they are often seen only in fragments. If very thick sections are cut they are so opaque that resolution is difficult.

The myoneural junctions from normal animals and from animals poisoned with E.605 were examined for retention of normal morphology, cleanness of staining, regularity of outline, retention of the normal number of neurofibrils and affinity for silver. In no respect did the poisoned end-plates differ from the controls.

### *Methylene blue method*

This method was tried after failure to demonstrate changes in myoneural junctions by the silver and gold methods. It was thought that methylene blue might share the properties of other quarternary ammonium compounds of interfering with nerve conduction, and also because it was a known inhibitor of cholinesterase (Rentz, 1940; Klein, 1944). It seemed likely that the selective uptake of methylene blue by nervous tissue might be related to its cholinesterase content and that the inactivation of cholinesterase by E.605 might result in an altered affinity for methylene blue, with failure to stain the end-plates as the likeliest possibility.

From a study of the myoneural junctions of normal animals and animals poisoned by E.605 no difference could be seen between the two groups, a conclusion completely in accord with that reached by the silver and gold methods. Although the myoneural junctions of both groups stained quite well there was considerable individual variation. This is somewhat difficult to illustrate owing to the difficulty of photography under high magnification of structures applied to the cylindrical surface of muscle fibres (see fig. 10) and to the fact that the contrast obtained with methylene blue is not nearly as good as with gold. However, some of the variability found in both normal and poisoned animals can be illustrated. Fig. 11 shows the picture often seen where the staining is chiefly of the hypolemmal axon. In fig. 12 the axon is practically unstained, whereas the gutter of the sole plate in which the hypolemmal axon lies is clearly stained, giving a picture closely resembling those obtained by Couteaux with Janus green. In fig. 13 the hypolemmal axon is darkly stained and the methylene blue is also deposited in granules on the sole-plate. These variations could not be controlled and obviously the method is not completely standardised.

Practical experience with the methylene-blue method has led to the acceptance of Schabadasch's general concept that methylene blue is taken up selectively only by living nervous tissue and that this occurs under anaerobic conditions. He attributes the selective uptake of methylene blue to the great capacity of nervous tissue for anaerobic glycolysis, the methylene blue acting as a hydrogen acceptor in the chain of carbohydrate metabolism. It is thus continuously reduced to the leuco-base. The regeneration of colour occurs when the experimental conditions are altered from anaerobic to aerobic. The reason for the firm localisation of methylene blue in the nervous tissue after reduction and then for some time after regeneration of full colour, is less well understood. Schabadasch attributes it to the accumulation of lactic acid following anaerobic glycolysis. This produces a change in *p*H to the acid side with a resulting binding of the basic methylene blue.

If it is accepted that methylene blue stains nervous tissue selectively because its oxidation reduction potential is such that it can act as a hydrogen acceptor, then the concept that staining is primarily due to the presence of cholinesterase in the tissue and that combination with methylene blue is due to its power to inhibit cholinesterase is no longer tenable. This has been confirmed by adding a potent, rapidly acting anticholinesterase agent to the staining solution at a concentration adequate to inhibit cholinesterase completely and observing that the myoneural junctions stained in the normal way. The compound tetra-ethyl pyrophosphate (TEPP) has been found by Aldridge (1950, personal communication) to produce 50 per cent. inhibition of true cholinesterase in 30 minutes at 37° C. at a molar concentration of $1{\cdot}2\times10^{-8}$. TEPP was added at a concentration of $10^{-5}$ molar to the staining solution immediately before injection and adequate staining of motor end-plates was obtained. The lack of correlation between methylene blue staining and cholinesterase content of myoneural junctions is confirmed by the work of Hillarp (1946), who found that nerves and myoneural junctions fully stained by methylene blue were still capable of transmitting impulses to muscle in the normal way.

## DISCUSSION

In E.605 poisoning two possible events have been considered, first that the myoneural junctions may be abnormal and second that the inactivation of cholinesterase might reduce all end-plates to a common physiological and morphological state. The junctions would then be expected to present a constant and uniform appearance. In the poisoned animals neither possibility was encountered. The normal structure was preserved along with the normal variability.

In view of the negative findings in E.605 poisoning it is instructive to examine the reports of the effect of other toxic agents on myoneural junctions. This investigation may be opened by considering the problems of acute beriberi and acute starvation, in which the lesions described by Woollard (1926-27) are often quoted in support of views associating myoneural lesions with disorders of function. It provides an example of the difficulty of assessing work in this field and the peculiar viability of ill-founded conclusions. In beriberi the almost immediate relief of severe symptoms by vitamin $B^1$ has long been a pathological curiosity. The degenerative changes in the brain, spinal cord and peripheral nerves that have been described at various times have always seemed inadequate as a primary cause of the symptoms. Tsunoda (1928) used the silver method to study the muscle and plates of pigeons with experimental beriberi and reported irregularity in the outline of the hypolemmal axon. Woollard with the gold and methylene blue methods found an early breakdown of the muscle end-plates of rats with beriberi and, surprisingly enough, thought that these lesions offered a more satisfactory explanation of the rapid recovery on treatment with vitamin $B^1$ than did the polyneuritis and spinal and cerebral lesions described by others. Woollard did not strengthen his case by describing similar changes in the end-plates of rats after short (6-7 day) periods of starvation, periods about four weeks less than the usual time required to produce experimental beriberi. This production of end-plate lesions by inanition has been controverted by Rogers *et al.* (1931), who reported that the finest ramifications of the hypolemmal axons were preserved on the surface of muscle fibres which had undergone complete hyaline necrosis in the condition of nutritional muscular dystrophy in guinea-pigs, now attributed to vitamin-E deficiency (Wolbach and Bessey, 1942). More recently Hines *et al.* (1943-44) could find no evidence that functional denervation resulted from prolonged acute inanition in rats and guinea-pigs. The modern view of the biochemistry and indefinite pathology of acute beriberi has been summarised by Peters (1949), who defines it as a " biochemical lesion " in which severe symptoms appear before any changes are visible under the microscope. We are not concerned here with the degenerative changes in nervous tissue that may arise in chronic beriberi but only with the lesion of acute beriberi as produced by Woollard in which the symptoms disappear in a few hours after treatment with vitamin $B^1$. It is now much easier to accept the idea that the symptoms arise from a disturbance of the pyruvate oxidase system rather than from a primary degeneration of the muscle end-plates.

Curare has been studied longer than other toxic agents which act at the myoneural junction and is the only one on which a substantial amount of pathological work has been done. But the conclusions of the various workers are inconsistent. Kühne (1860) thought that the unstained reptilian end-plate was rendered more distinct when poisoned with curare. Miura (1886), using the gold chloride method, found some atrophy of the end-plates in lizards after 40 days of poisoning, but no change in frogs. Herzen and Odier (1904) also employed the gold chloride method with frogs and thought that treatment with curare produced irregularity in the outline of the digitations. To this inconclusive earlier work can be added some recent but conflicting reports. Rojas *et al.* (1939), applying both the gold and silver methods, describe in curarised lizards an enlargement of the end-plates and an increased prominence of the hypolemmal axon. Carey (1944*a*), using the gold chloride method with chameleons, reached the opposite conclusion that curare caused a retraction of the end-plate. In addition he reported an increased chrysophilia and accretion of the granules of Kühne. Dublin (1944), on the other hand, could find no remarkable variation in the muscle end-plates of curarised rabbits stained by the gold method. This last conclusion is supported by the work of King (1945), who measured the muscle end-plates, stained by the gold chloride method, of normal and curarised rats and could find no difference in size or structure.

There is much less information on the histological changes in poisoning by other toxic agents acting at the myoneural junction. The appearance of end-plates in experimental poisoning of guinea-pigs by botulinus toxin has been reported by Guyton and MacDonald. The toxin was injected intramuscularly so as to produce local paralysis and the affected end-plates were examined by the gold chloride method after varying periods of paralysis from four days to seven months. They formed the guarded opinion that it could not be concluded that there were any definite structural changes in the poisoned end-plates, although these were not entirely normal in the earlier stages of poisoning. They expressed surprise that poisoning and paralysis of over six months' duration could occur without morphological change in the myoneural junctions.

Internal evidence throws some doubt on the conclusions reached by Kura and Kamesawa (1928) on the myoneural changes in tetanus. They injected tetanus toxin subcutaneously in mice and with the silver method found coarseness of outline of the hypolemmal axon, thickening of nerve fibres and irregularity in the silver staining. These findings are strikingly similar to changes found in beriberi in pigeons and reported from the same laboratory at the same time (Tsunoda). Indeed, Tsunoda in his paper attributes very similar changes in the end-plates of the normal rat to delay in fixation.

The work quoted so far certainly does not justify the conclusion that morphological changes can be experimentally induced in muscle end-plates, but little mention has been made of the work of Carey, the chief proponent of this view. In 1941*a* Carey stated briefly that the pleomorphism of the end-plate is a consequence of its amœboid movement on the muscle fibril and, perhaps less clearly than in later papers, that the variations in the granularity of the sole-plate are evidence of different phases in the periodic activity of the secretory mechanism of the end-plates. Over the next eight years he and his colleagues published a series of papers supporting these hypotheses. The criticism of the gold chloride method detailed in an earlier part of this paper applies to Carey's work as a whole. In his papers there is no real attempt to define the normal variation, the position adopted being that this is a problem " comparable to the study of the various complex forms of the waves that compose breakers along a diversified coastline " (Carey *et al.*, 1946*b*, p. 184). Despite this fundamental difficulty Carey and his associates were able to describe the production of characteristic changes in myoneural junctions by many different ways—by electrical shocks and the inhalation of carbon dioxide (Carey, 1941*a* and *b*, 1942), by the action of a number of drugs, including curare, quinine, physostigmine and acetylcholine (Carey, 1944*a*), and D.D.T. (Carey *et al.*, 1946*a*), by the injection of lactic acid (Carey and Massopust, 1944), in human poliomyelitis (Carey *et al.*, 1944*a*) and in experimental poliomyelitis in monkeys (Carey, 1943; Carey, 1944*b*), following hæmorrhagic shock (Carey *et al.*, 1944*b*), traumatic shock (Carey *et al.*, 1945*a*, Carey *et al.*, 1945*b*) and thermal shock (Carey *et al.*, 1946*b*), after nerve section (Carey *et al.*, 1946*c*), and after disuse (Carey *et al.*, 1948). This formidable series of papers is characterised by beautiful illustrations and many novel views on the structure and particularly the function of nerve and muscle. Some of these opinions can be illustrated by reference to the evidence which " appears to support the claim that there are morphologic as well as chemical transmitters of normal and abnormal nerve impulses " (p. 1227) and that motor end-plates " appear to be biologic jet-pumps that discharge either normal or abnormal neurogenic substances into the myoplasm of the muscle fibers " (Carey *et al.*, 1946*c*, p. 1226), and the view that cross striations of voluntary muscle are " structural expressions of the changeable mechanical energy of explosive pressure that accompanies the capillary chemistry of metabolism " (Carey *et al.*, 1946*b*, p. 196).

The acceptance of the view that toxic agents can produce characteristic changes in myoneural junctions (McIntyre, 1947; Marrazzi, 1948; Lehman, 1949; Cowdry, 1950, and others) has usually been based on one or other of Carey's papers and not on a critical assessment of the available evidence, which, taken as a whole, does not support either the hypothesis that morphological lesions have been produced by a variety of toxic agents, or the ancillary suggestion that the great variability of myoneural junctions in normal animals is the consequence of their varying physiological states. Two main reasons can be given to explain the contradictory findings which have been recorded. The depth and selectivity of the staining cannot be adequately controlled, a point that has been emphasised by Bremer (1936) and Gutmann and Young (1944). Many workers have failed to establish the normal range of variation and have made the error of attributing to the action of specific toxic agents what are really normal appearances.

The present work records the failure to find pathological lesions in animals poisoned by an anticholinesterase drug. It provides additional evidence of the inadequacy of the three methods, the gold chloride method of Löwit, the Bielschowsky-Gross silver method and the methylene blue method of Ehrlich as means of investigating the histological aspects of poisoning by agents known to act at the myoneural junctions. But the possibility of using the microscope to study the effect of toxic agents on myoneural junctions is not excluded. Perhaps such methods as phase contrast microscopy, an examination of the mitochondria of the sole plate on the lines of Noël and Pommé, the use of the Janus-green method of Couteaux, or a study of the site of activity of cholinesterase by methods such as those of Gomori (1948) and Koelle and Friedenwald (1949) may offer more promising lines of investigation. But such techniques are unlikely to be used until the inadequacy of the more usual methods is fully appreciated and some of the generally accepted reports more critically assessed. This paper is directed towards this end, with the definite statement that the production of pathological lesions of myoneural junctions by toxic agents such as curare, botulinus toxin, tetanus toxin and anticholinesterases has not yet been satisfactorily demonstrated.

## SUMMARY

1. The histological structure of myoneural junctions of the albino rat was examined in the normal animal and also in the rat poisoned by the anticholinesterase drug, para-nitrophenyl diethyl thiophosphate (E.605).

2. The gold chloride method, the ammoniacal silver method and the methylene blue method were used to demonstrate the myoneural junctions.

3. No consistent changes that could be attributed to the action of E.605 were found.

4. The reports in the literature of structural changes in myoneural junctions produced by other toxic agents such as curare and botulinus toxin are critically assessed.

5. The conclusion is reached that the production of visible pathological lesions in the myoneural junctions by such toxic agents has not been satisfactorily demonstrated.

6. The inconsistencies in the literature are accounted for by the extreme variability of the normal structure and by the fact that reproducible results have not been obtained with the usual histological methods for myoneural junctions.

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# THE ANTIDIURETIC HORMONE OF THE POSTERIOR LOBE OF THE HUMAN HYPOPHYSIS

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RECENT evidence suggests that neurohypophysial function is modified in a variety of clinical and experimental conditions. It seemed of interest, therefore, to investigate the hormone content of human posterior pituitary glands obtained from post-mortem material which included instances of diseases in which disturbances of the metabolism of water are known to occur.

A difficulty inherent in such an investigation is the delay between the death of the patient and the removal of the material for analysis. In the present series the glands were removed 10 to 25 hours after death and it is assumed that no marked losses of activity occurred during these intervals. This assumption appears justified in view of the finding of Heller and Zaimis (1949) that the antidiuretic and oxytocic potency of extracts of human posterior lobes, removed 7 to 40 hours after death, showed no correlation with the length of time which the glands had been kept *in situ*.

## MATERIAL AND METHODS

The antidiuretic potency of extracts of 58 human hypophyses was estimated; the age of the subjects ranged from 16 to 78 years; 33 were males and 25 were females.

*Preparation of extracts.* The posterior pituitary lobes were carefully dissected as soon as possible after death and immediately ground in a mortar with 0·9 per cent. NaCl solution. One ml. of saline was allowed per mg. of gland.

*Method of antidiuretic assay.* Burn's (1931) method was used. Water and food was withheld from the rats for 12 hours before the experiment. The output of urine was calculated as percentage of the water administered. Readings were taken every 15 minutes for a period of three hours. The neurohypophysial extracts were injected immediately after they had been prepared. The standard dose was 0·5 ml. of extract (equivalent to 0·5 mg. of neurohypophysial tissue) per 100 g. rat. Control experiments were made with the same volume of 0·9 per cent. NaCl solution only and with extracts of indifferent nervous tissues (cerebral and cerebellar cortex, pineal body) in the same doses, *i.e.* 0·5 ml. (0·5 mg.) per 100 g. rat.

*Statistical evaluation of results.* Standard errors (S.E.) were calculated as $\sqrt{\frac{Sx^2}{n(n-1)}}$ and Student's " t " by means of the following formula:

$$t = \frac{\bar{x}-\bar{y}}{\sqrt{\left(\frac{Sx^2+Sy^2}{n_1+n_2-2}\right)\times\left(\frac{1}{n_1}+\frac{1}{n_2}\right)}};$$

differences between two groups were considered significant when " $t$ " $>2{\cdot}2$.

## RESULTS

Table I compares the antidiuretic effect of neurohypophysial extracts with the results obtained with the control solutions. It shows the high potency of the posterior pituitary extracts and the absence of antidiuretic activity in the control extracts. No relation could be observed between hormone content and sex but remarkable differences appeared when the post-mortem material was grouped according to the disease from which the patients had suffered (table II). The "normal" group comprised glands from cases in which no abnormalities of the metabolism of water had been evident. The causes of death in this group were malignant growth, tuberculosis, sepsis, meningitis, abortion, leukæmia and gastric ulcer. The extracts from the gland of such cases showed a fairly uniform activity which was considered as "normal" and which served as basis of comparison for the results obtained in the other groups. The interest of the

TABLE I

*Mean effect on water diuresis of rats produced by saline, nervous tissue controls and neurohypophysial extracts (for dosage see text)*

| | No. of extracts tested | Percentage of water administered excreted at indicated minute | | | S.E. (of values at 180 min.) | "t" |
|---|---|---|---|---|---|---|
| | | 60 | 120 | 180 | | |
| Saline . . . . | 4 | 23·4 | 60·7 | 66·9 | ±3·60 | ... |
| Control extracts of nervous tissue | 10 | 13·2 | 46·3 | 55·0 | ±7·81 | 0·07 |
| Neurohypophysial | 58 | 4·0 | 15·3 | 22·2 | ±1·67 | 7·05 |

TABLE II

*Mean effect on water diuresis of rats produced by neurohypophysial extracts from post-mortem material grouped according to morbid conditions (for dosage see text)*

| Morbid condition | No. of cases | Percentage of water administered excreted at indicated minute | | | S.E. (of values at 180 min.) | "t" |
|---|---|---|---|---|---|---|
| | | 60 | 120 | 180 | | |
| "Normal" . . . | 18 | 7·1 | 21·1 | 33·7 | ±2·65 | ... |
| Cirrhosis . . . | 11 | 2·4 | 7·6 | 16·7 | ±2·79 | 3·6 |
| Hypertension . . | 10 | 3·2 | 11·1 | 18·3 | ±2·91 | 3·6 |
| Cardiac œdema . . | 9 | 2·2 | 8·4 | 14·0 | ±2·19 | 4·3 |
| Diabetes mellitus . . | 4 | 3·4 | 6·6 | 14·1 | ±2·10 | 3·1 |
| Eclampsia . . . | 1 | 1·4 | 3·4 | 11·9 | ... | ... |
| Addison's disease . . | 1 | 6·1 | 11·2 | 13·8 | ... | ... |
| Brain tumour . . | 2 | 8·5 | 15·6 | 17·5 | ... | ... |
| Diabetes insipidus . . | 1 | 8·5 | 49·1 | 62·3 | ... | ... |
| Hæmochromatosis . . | 1 | 12·1 | 44·3 | 56·8 | ... | ... |

table lies in the cases of renal and essential hypertension, cirrhosis with ascites, cardiac failure with œdema, diabetes mellitus, eclampsia and Addison's disease. It will be seen that the antidiuretic potency of the extracts obtained from the posterior pituitary glands of these cases was significantly higher than the mean effect exerted by the extracts classed as "normal." A higher antidiuretic activity could also be demonstrated in two cases of brain tumour. Finally a case of diabetes insipidus and one of hæmochromatosis deserve attention. Practically no antidiuretic effect was observed after the injection of the pituitary extract from the case of diabetes insipidus in which the posterior lobe appeared to be replaced by tuberculous granulation tissue (compare with the results of control injections, table I). The assay in the case of hæmochromatosis, in which the posterior lobe was strongly pigmented, also gave low results.

## Discussion

The antidiuretic hormone in the posterior pituitary gland was found to be significantly increased in diseases which are known to be associated with marked retention of water, such as severe cirrhosis with ascites, cardiac œdema, arterial hypertension, eclampsia and Addison's disease. Similar results were obtained in diabetes mellitus, *i.e.* in a condition in which abnormalities of electrolyte and water metabolism have been shown to occur even before the onset of coma (Blum, 1928 ; Atchley *et al.*, 1933 ; Mach, 1946). In striking contrast to these findings was the very low hormone content in a case of diabetes insipidus which is in accordance with the recently published findings of Hewer and Heller (1949), and in a case of massive pigmentary infiltration of the neurohypophysis.

An increase of antidiuretic activity in body fluids is said to occur in liver cirrhosis (Malaguzzi-Valeri and Mininni-Montesano, 1941 ; Ralli *et al.*, 1945 ; Leslie and Ralli, 1947 ; Drill and Frame, 1948 ; Hall *et al.*, 1949), essential hypertension (Malaguzzi-Valeri and Mininni-Montesano, 1942 ; Ellis and Grollman, 1949), eclampsia (Anselmino and Hoffmann, 1931 ; Teel and Reid, 1939 ; Ham and Landis, 1942), chronic nephritis (Bohn and Hahn, 1933 ; Coester, 1933-34 ; Robinson and Farr, 1940-41), acute hepatitis (Malaguzzi-Valeri *et al.*, 1949), normal pregnancy (Krieger and Kilvington, 1946 ; Colucci and Siliotti, 1948), and in hunger œdema (Gopalan, 1950). So far it has not been proved that these antidiuretic activities derive from the neurohypophysis. In fact, the evidence is, if anything, to the contrary (Ham and Landis, 1942 ; Dicker, 1950). However, it will be noted that the conditions enumerated include some in which an increased antidiuretic potency per mg. fresh neurohypophysial tissue could be demonstrated. A correlation between the increased antidiuretic titre of body fluids and the increased hormone content in the gland is thus suggested.

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| | | 60 | 120 | 180 | | |
| "Normal" . . . | 18 | 7·1 | 21·1 | 33·7 | ±2·65 | ... |
| Cirrhosis . . . | 11 | 2·1 | 7·6 | 16·7 | ±2·79 | 3·6 |
| Hypertension . . | 10 | 3·2 | 11·1 | 18·3 | ±2·01 | 3·6 |
| Cardiac œdema . . | 9 | 2·2 | 8·4 | 14·0 | ±2·19 | 4·3 |
| Diabetes mellitus . . | 4 | 3·4 | 6·6 | 14·1 | ±2·10 | 3·1 |
| Eclampsia . . . | 1 | 1·4 | 3·1 | 11·9 | ... | ... |
| Addison's disease . . | 1 | 6·1 | 11·2 | 13·8 | ... | ... |
| Brain tumour . . | 2 | 8·5 | 15·6 | 17·5 | ... | ... |
| Diabetes insipidus . . | 1 | 8·5 | 49·1 | 62·3 | ... | ... |
| Hæmochromatosis . . | 1 | 12·1 | 41·3 | 56·8 | ... | ... |

612 . 015 . 31 ($Sr^{89}$, $Sr^{90}$, $Y^{90}$) : 618 [. 2+ . 63 (Cuneus)
612 . 015 . 31 ($Sr^{89}$, $Sr^{90}$, $Y^{90}$) : 616—053 . 31 (Cuneus)

# THE RETENTION OF RADIOACTIVE STRONTIUM AND YTTRIUM ($Sr^{89}$, $Sr^{90}$ AND $Y^{90}$) IN PREGNANT AND LACTATING RABBITS AND THEIR OFFSPRING *

Barbara Kidman, Margaret L. Tutt † and Janet M. Vaughan

*From the Department of Pharmacology and the Nuffield Department of Medicine, Oxford*

(Plate XLII)

An investigation of the metabolism of $Sr^{89}$, $Sr^{90}$ and $Y^{90}$ in young and adult rabbits has been reported (Kidman *et al.*, 1950). A further investigation of the effect of diet and of the time of injection in relation to delivery on the retention of the same isotope mixture in pregnant and lactating rabbits and their offspring is now described. Excretion studies, except in the milk, on the present group of animals were impractical owing to the presence of a nest and its contents.

## Experimental procedures

### (1) *The effect of diet on retention*

The effect of diet was studied in three groups of rabbits on the same diets as previously recorded ·—(i) a low calcium intake, (ii) a medium calcium intake, (iii) a high calcium intake (Kidman *et al* ). It proved impossible to breed rabbits without giving them material with which to make a nest 3 4 days before delivery. In the present experiments with one exception hay was used which contains 1 6 per cent. of calcium oxide. It was hoped to include a second group of low calcium rabbits nested on straw which has a lower calcium content of 0 6 per cent. Unfortunately it is extremely difficult to breed from rabbits in the low calcium group and only one rabbit nested on straw is included here. An attempt to use cotton wool was disastrous as the mother ate the cotton wool and died with a ruptured stomach. The calcium deficient mother eats hay or straw with avidity and the baby rabbits start to nibble any time after the first week so both mothers and sucklings had a somewhat increased calcium intake over a short period. The radiograms of the bones of weanling rabbits from mothers on a low calcium diet who had access to hay suggest that though there was a probable increase in calcium intake it had little obvious effect on calcification (Kidman *et al.*). Radiographic examination of the fœtuses also showed a marked difference dependent on the diet of the mother (fig. 1).

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* Work undertaken on behalf of the Protection Sub Committee of the Medical Research Council Committee on Medical and Biological Applications of Nuclear Physics

† Nuffield Graduate Assistant

In order to test the possible effect of the temporary provision of hay on the intake of strontium in rabbits on a low calcium diet three six-months-old virgin rabbits were given hay *ad lib.* in addition to their oats and potatoes for seven days before the intravenous injection of the routine dose of strontium. They were then placed in metabolism cages on the same diet with access to hay and killed at the end of 9 days.

The rabbits used for dietary studies were given a single injection of approximately 25 μc. of the isotope mixture per kg. body weight into the marginal vein of the ear, in some cases with and some without carrier 9 days before delivery.

(a) *Pregnant rabbits.* For purposes of direct comparison with the unencumbered adult rabbit the pregnant rabbit should have been 6 months old. It was not always possible to use rabbits of exactly this age owing to difficulty in keeping a large enough stock. The average age of the pregnant rabbits receiving an injection 9 days before delivery was 7 months, varying from 6-8 months with one exception noted in the text. All rabbits were killed the day delivery was expected. In some cases the young were born the night before and suckling had occurred. This was ignored in the rabbits injected 9 days before delivery as there is evidence that excretion in the milk falls to a low level within 48 hours of injection (Erf and Pecher, 1940).

(b) *Lactating rabbits.* Rabbits used for dietary studies received a single injection of 25 μc. of the isotope solution per kg. body weight in the marginal vein of the ear, in some cases with and some without carrier, 7 days after delivery. They were killed together with their litters 9 days later.

## (2) *Effect of time of injection on retention*

(a) *Pregnant rabbits.* Three rabbits on a medium calcium diet were given 100 μc. per kg. 38 or 50 days before delivery. They had an average age of 6 months, varying from 5-7 months. Six rabbits varying in age from 6-12 months with an average age of 7 months on different diets received an injection of 25 μc. per kg. 24 hours before delivery. Both groups were killed the day the litter was due. If the litter had been delivered the stomachs of the fœtuses were dissected out and the strontium content of the milk determined.

(b) *Lactating rabbits.* Two litters from normal rabbits were transferred at birth to mothers injected 38 days previously with 100 μc. of strontium and subsequently mated. One mother was aged 5 months and the other 6 months at the time of injection. Their own litters were destroyed. The normal fœtuses were thus suckled by mothers whose bones contained radioactive strontium and yttrium.

## (3) *Excretion in the milk*

Eight lactating rabbits on a medium calcium diet were used to study the excretion of strontium in the milk. Two to 10 days after delivery 6 of these rabbits received a single intravenous injection of isotope mixture containing approximately 15 μc. of strontium per kg. body weight. They were subsequently milked by hand or with an electric machine for 1 to 5 days. No attempt to strip the rabbit at each milking was made: amounts varying from 0·5 to 2 ml. were withdrawn and data were expressed as the percentage of strontium injected per ml. of milk. After milking the mother was returned to her litter which was reduced to 1-4 in number, except in two cases where the litter died. Two rabbits were injected 38 days before delivery with solutions containing approximately 100 μc. of strontium and the strontium content of their milk was estimated after delivery. In three instances before skill in milking had been achieved one or more doses of extract of the posterior lobe of the pituitary

STRONTIUM AND YTTRIUM ISOTOPES

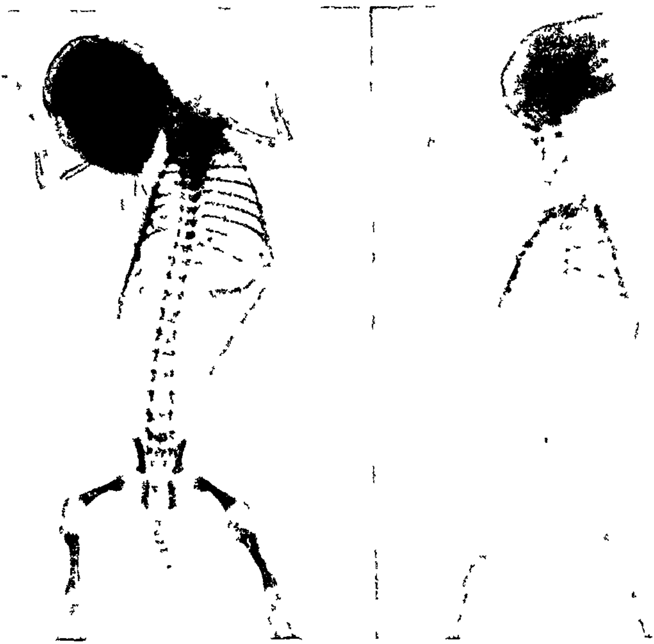

FIG 1

(*a*) Full term fœtus mother fed on a medium calcium diet

(*b*) Full term fœtus mother fed on a low calcium diet

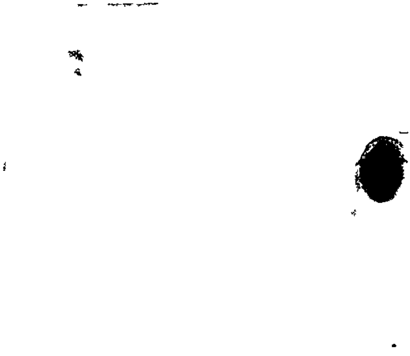

FIG 4

(*a*) 22 day old fœtus mother fed on a medium calcium diet

(*b*) 20 day old fœtus mother fed on a medium calcium diet

were given. In two of the rabbits blood samples were taken at the same time as milk samples. Blood samples were also taken from 4 non-lactating rabbits after injection.

### (4) *Preparation of samples and measurement of radioactive elements*

The bones of mothers and sucklings were dissected out and dissolved in concentrated nitric acid as previously described (Kidman *et al.*). The fœtuses were dissolved in a similar manner. The strontium content was expressed per whole litter, per individual fœtus and, when the fœtuses were weighed, per g. of fœtus. In the case of the milk 0·1 ml. was measured on to a nickel specimen disc and dried over a water bath. One to five such samples were taken from the milk obtained at any one time and the activity measured with a G.E.C. counter type G.M. 4, the average figure being recorded. In one series parallel estimations were made using diluted milk in a type M. 6 counter (Veall, 1948). This may prove the more satisfactory technique but in the present paper only the results with the dry method are recorded.

Blood was withdrawn from the marginal vein of the ear, diluted with distilled water and activity estimated in a liquid counter type M. 6 (Veall).

### (5) *Estimation of radioactive elements*

The methods used for estimation of radioactive strontium and yttrium in the biological material were the same as those described by Kidman *et al.* In the earlier experiments of which a preliminary report has already been published (Tutt and Vaughan, 1949) the importance of allowing material to reach radioactive equilibrium for a correct estimation of strontium content was not appreciated. These results are not included here.

Counts were made as soon as possible after collection of samples and again after radioactive equilibrium was reached on all samples except the blood of rabbits 73 and 68 which were measured only once shortly after withdrawal. The decay curves on the blood of rabbit 257 and rabbit 284 showed that the maximum change in activity was only 20-30 per cent.; therefore it appears justifiable to include all the data on blood levels.

## Results

### Retention of strontium

### (a) *Rabbits on a low calcium diet given access to hay*

The three virgin rabbits on a low calcium diet given access to hay for 7 days before injection and subsequently for 9 days had a mean retention of 20·9 per cent. of the strontium injected. The mean excretion in the urine was 33·8 per cent. and in the fæces 32·9 per cent. The mean total recovery was 87·5 per cent. As previously reported, virgin rabbits of the same age on a low calcium diet without access to hay retained 57·7 per cent. of the strontium injected, excreting only 5·6 per cent. in their urine (Kidman *et al.*).

### (b) *Pregnant mothers*

(i) *Injection* 38-50 *days before delivery.* Two rabbits on a medium calcium diet injected 38 days before delivery had a mean retention of 12·1 per cent. of the injected dose and 0·06 per cent. per g. of bone.

One rabbit injected 50 days before delivery had a retention of 7·4 per cent. of the injected dose, representing 0·03 per cent. per g. of bone (table I).

(ii) *Injection 9 days before delivery.* The strontium retained by pregnant rabbits injected 9 days before delivery on a low calcium diet was 36·3 per cent., on a medium calcium diet was 12·8 per cent. and on a high calcium diet was 10·2 per cent. of the strontium injected (table I). The retention per g. of bone was 0·2 per cent. on a low calcium diet, 0·07 per cent. on a medium calcium diet and 0·05 per cent. on a high calcium diet.

Table I

*Effect of diet and time of injection on the retention of radioactive strontium in pregnant rabbits*

| Time of injection | Carrier | Percentage of injected strontium retained in bone | | | | | | Percentage of injected strontium retained per g. bone | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | No. in litter | Low calcium diet | No. in litter | Medium calcium diet | No. in litter | High calcium diet | Low calcium diet | Medium calcium diet | High calcium diet |
| 24 hours | + | 1 | 28·8 | 8 | 14·4 | 4 | 10·1 | 0·16 | 0·07 | 0·06 |
| | 0 | ... | ... | 7 | 14·2 | 5 | 19·0 | ... | 0·07 | 0·11 |
| | 0 | ... | ... | ... | ... | 4 | 12·2 | ... | ... | 0·07 |
| 9 days | + | 2 | 50·8 | 4 | 13·1 | 5 | 9·2 | 0·31 | 0·07 | 0·05 |
| | 0 | 1 | 23·8 | 7 | 12·3 | 9 | 12·9 | 0·12 | 0·06 | 0·06 |
| | 0 | 2 | 34·3 | 2 | 12·8 | 4 | 8·5 | 0·18 | 0·07 | 0·05 |
| 38 days | + | ... | ... | 8 | 13·6 | ... | ... | ... | 0·07 | ... |
| | + | ... | ... | 3 | 10·6 | ... | ... | ... | 0·05 | ... |
| 50 days | 0 | ... | ... | 8 | 7·4 | ... | ... | ... | 0·03 | ... |

(iii) *Injection 24 hours before delivery.* When the injection was made 24 hours before delivery the average retention in the bones of the mother on a medium calcium diet was 14·3 per cent., and on a high calcium intake was 13·8 per cent. Only one mother on a low calcium diet was available and she was 12 months old. Retention was 28·8 per cent. of that injected. Retention per g. of bone was 0·16 per cent. on a low calcium diet, 0·07 per cent. on a medium calcium diet and 0·08 per cent. on a high calcium diet (table I).

### (c) *Fœtuses*

(i) *Injection of mother 38-50 days before delivery.* The mean retention in the litter of two mothers injected 38 days before delivery was 0·03 per cent., the mean retention per fœtus was 0·005 per cent. or expressed per g. of fœtus 0·0002 per cent. The retention in the litter of a mother injected 50 days before delivery was 0·04 per cent., 0·005 per cent. per fœtus or 0·00015 per cent. per g. (table II).

(ii) *Injection of mother 9 days before delivery* The litters from mothers injected 9 days before delivery on a low calcium diet contained a mean of 2 4 per cent of the injected dose, on a medium calcium diet 0 78 per cent and on a high calcium diet 1 1 per cent If retention is expressed per fœtus on a low calcium intake each fœtus retained

TABLE II

*Effect of diet and time of injection on the retention of radioactive strontium in the litters of rabbits on different diets*

| Time of injection | Carrier | Percentage of injected strontium retained per litter | | | | | | Percentage of injected strontium retained per fœtus | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | No in litter | Low calcium diet | No in litter | Medium calcium diet | No in litter | High calcium diet | Low calcium diet | Medium calcium diet | High calcium diet |
| 24 hours | + | 1 | 4 2 | 8 | 13 1 | 4 | 4 1 | 4 2 | 1 6 | 1 0 |
| | 0 | | | 7 | 9 5 | 5 | 7 1 | | 1 4 | 1 4 |
| | 0 | | | | | 4 | 6 0 | | | 1 5 |
| 9 days | + | 2 | 2 6 | 4 | 0 80 | 5 | 0 74 | 1 3 | 0 20 | 0 15 |
| | 0 | 1 | 0 49 | 2 | 0 53 | 4 | 0 62 | 0 49 | 0 26 | 0 16 |
| | 0 | 2 | 4 1 | 7 | 1 0 | 9 | 2 0 | 2 0 | 0 15 | 0 22 |
| 38 days | + | | | 8 | 0 05 | | | | 0 006 | |
| | + | | | 3 | 0 01 | | | | 0 005 | |
| 50 days | 0 | | | 8 | 0 04 | | | | 0 005 | |

1 26 per cent of the injected dose, on a medium calcium intake 0 2 per cent, and on a high calcium intake 0 18 per cent On a medium and high calcium diet the amount retained per litter was affected by the number in the litter, being greater the larger the litter. The number of fœtuses in the low calcium litters never exceeded two so no conclusions on the effect of litter size on total retention in this group can be made (table II)

(iii) *Injection of mother 24 hours before delivery* Retention in the fœtuses from mothers injected 24 hours before delivery exclusive of what was found in the stomach was greater than in those injected 9 days before delivery On a low calcium intake one litter retained 4 2 per cent of the injected strontium, on a medium calcium intake two litters retained an average of 11 3 per cent and on a high calcium intake the mean retention in three litters was 5 7 per cent Expressed per fœtus the mean retention on a low calcium intake was 4 2 per cent, on a medium calcium intake 1 5 per cent and on a high calcium intake 1 3 per cent The amount in the fœtal stomachs varied from 0 3 to 0 6 per cent of the injected dose (table II)

In some cases the percentage retained per g of fœtus was also compared with the percentage retained per g of maternal tissue, with a view to determining whether there was any concentration in the fœtus. The retention per g. of tissue of the fœtuses of mothers injected 24 hours before delivery was greater than per g. of the mothers' tissue (table III). *This was not the case in the fœtuses of mothers injected* 9 days or 38 days before delivery.

TABLE III

*Mean values for percentage of strontium retained expressed per g. of whole tissue in mother and fœtus*

| | Mother | | | Fœtuses | | |
|---|---|---|---|---|---|---|
| Time of injection | Low calcium diet | Medium calcium diet | High calcium diet | Low calcium diet | Medium calcium diet | High calcium diet |
| 24 hours | 0·018 | 0·0050 | 0·0066 | 0·085 | 0·040 | 0·028 |
| 9 days | 0·019 | 0·0054 | 0·0046 | ... | ... | 0·0035 |
| 38 days | ... | 0·0065 | ... | ... | 0·00018 | ... |
| 50 days | ... | 0·0032 | ... | ... | 0·00015 | ... |

## (d) *Lactating mothers*

(i) *Injected 7 days after delivery.* The mean retention of injected strontium in three lactating rabbits on a low calcium intake with access to hay was 13·4 per cent. When straw, which has a lower calcium content, was substituted for hay in one rabbit the retention was 26·1 per cent. On a medium calcium diet the mean retention was 6·6 per cent. and on a high calcium diet 5·7 per cent. Results expressed as percentage retained per g. of bone were 0·07 for rabbits on a low calcium diet with access to hay, 0·15 for the rabbit with access to straw, and 0·03 for rabbits on a medium and a high calcium diet (table IV).

TABLE IV

*Retention, in lactating rabbits on different diets, of radioactive strontium injected before and after delivery*

| Time of injection | Carrier | Percentage of injected strontium retained in bone | | | | | | Percentage of injected strontium retained per g. of bone | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | No. in litter | Low calcium diet | No. in litter | Medium calcium diet | No. in litter | High calcium diet | Low calcium diet | Medium calcium diet | High calcium diet |
| 7 days after delivery | + | 4 | 17·1 | 6 | 6·4 | 4 | 8·6 | 0·09 | 0·03 | 0·04 |
| | + | ... | ... | 3 | 6·6 | 8 | 2·9 | ... | 0·03 | 0·01 |
| | 0 | 3 | 9·3 | 2 | 6·9 | 3 | 5·5 | 0·05 | 0·03 | 0·03 |
| | 0 | 5 | 13·9 | ... | ... | ... | ... | 0·08 | ... | ... |
| | +* | 3 | 26·1 | ... | ... | ... | ... | 0·15 | ... | ... |
| 38 days before delivery | + | ... | ... | foster 2 | 8·4 | ... | ... | ... | 0·04 | ... |
| | + | ... | ... | 4 | 12·2 | ... | | ... | 0·06 | ... |

* Nested in straw.

(ii) *Injected* 38 *days before delivery.* The mean retention in two rabbits, aged 5-6 months used as foster mothers, on a medium calcium diet and injected 38 days before delivery and killed 9 days after delivery was 10·3 per cent. or 0·05 per cent. per g. of bone (table IV).

### (e) *Suckling rabbits*

(i) *Mother injected* 7 *days after delivery.* The strontium retained by suckling rabbits on a low calcium diet with hay available varied considerably. In three litters retention varied from 30·7 to 14·2 per cent.; it did not appear influenced by the size of the litter; the mean retention was 20·7 per cent. or 0·22 per cent. per g. of bone. In the one litter where the mother was nested on straw the percentage retention in the sucklings was 12·7 only, *i.e.* less than that of rabbits nested on hay. The percentage per g. of bone was 0·3 which, though greater than the mean retention when hay was given, is no greater than in one of the litters. On a medium calcium intake the suckling rabbits retained 10·5 per cent. and on a high calcium intake 12·7 per cent. of the injected strontium. The percentage retention per g. of bone was 0·10 per cent. on a medium calcium diet and 0·09 per cent. on a high calcium diet (table V).

TABLE V

*Retention of radioactive strontium in suckled rabbits on different diets, when the mother was injected before or after delivery*

| Time of injection of mother | Carrier | Percentage of injected strontium retained in bones of litter | | | | | | Percentage of injected strontium retained per g. bone | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | No. in litter | Low calcium diet | No. in litter | Medium calcium diet | No. in litter | High calcium diet | Low calcium diet | Medium calcium diet | High calcium diet |
| 7 days after delivery | + | 4 | 30·7 | 6 | 15·0 | 4 | 16·6 | 0·33 | 0·11 | 0·12 |
| | + | ... | ... | 3 | 7·2 | 8 | 9·3 | ... | 0·06 | 0·05 |
| | 0 | 3 | 14·2 | 2 | 9·4 | 3 | 12·2 | 0·15 | 0·13 | 0·10 |
| | 0 | 5 | 17·1 | ... | ... | ... | ... | 0·17 | ... | ... |
| | +* | 3 | 12·8 | ... | ... | ... | ... | 0·30 | ... | ... |
| 38 days before delivery | + | ... | ... | 2 | 0·36 | ... | ... | ... | 0·007 | ... |
| | + | ... | ... | 4 | 0·15 | ... | ... | ... | 0·002 | ... |

* Nested in straw.

(ii) *Lactating foster mothers injected* 38 *days before delivery.* The mean retention in the sucklings of two litters who were fostered on mothers injected 38 days before delivery and killed nine days after birth was 0·26 per cent. of that injected or 0·005 per cent. per g. of bone (table V).

### Excretion of injected strontium in the milk

#### (a) *Mother injected 38 days before delivery*

The activity of a sample of milk withdrawn two to three days after delivery from two rabbits injected 38 days before delivery was so slight as to be only just above background and no accurate estimate of the strontium content could be made. The activity of the blood withdrawn from rabbits 50 days after injection was so low that calculation of the strontium content was not justified.

#### (b) *Mother injected 2-10 days after delivery*

In rabbits numbers 235 and 257 the litters died before milking began. The other four rabbits were returned to their litters from time to time.

TABLE VI

*Percentage of injected strontium in milk and blood of individual rabbits*

| Time after injection | Percentage of injected strontium per ml. of milk in individual rabbits | | | | | | Percentage of injected strontium per ml. of blood | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 235 | 237 | 258 | 232 | 257 | 284 | 257 | 254 | 73 | 68 |
| 5 minutes | ... | ... | ... | ... | ... | ... | 0·17 | ... | ... | ... |
| 10 ,, | ... | ... | ... | ... | 0·0053 | ... | ... | 0·11 | 0·16 | 0·19 |
| 10 mins.-1 hr. | ... | ... | ... | 0·016 | ... | 0·0052 | ... | ... | ... | ... |
| 1-2 hrs. | 0·021$^p$ | ... | ... | ... | 0·035 | 0·014 | 0·044 ... | 0·045 | ... | ... |
| 2-3 ,, | ... | ... | 0·091$^p$ | 0·026 | 0·067 | 0·068 | 0·039 | 0·029 | 0·058 | 0·04 |
| 3-4 ,, | ... | ... | ... | ... | 0·085 | 0·047 | 0·056 | 0·025 | ... | ... |
| 4-5 ,, | 0·033 | 0·41$^{pppp}$ | ... | 0·041 | 0·085 | ... | 0·014 | ... | 0·0095 | 0·01 |
| 5-6 ,, | ... | ... | 0·12 | 0·068 | ... | 0·059 | ... | 0·011 | ... | 0·00 |
| 6-7 ,, | 0·060$^p$ | 0·30$^p$ | ... | ... | ... | ... | 0·0087 | ... | ... | ... |
| 7-8 ,, | ... | ... | 0·21 | ... | 0·087 | ... | ... | ... | ... | ... |
| 8-9 ,, | ... | ... | ... | ... | 0·086 | 0·062 | 0·0067 | 0·0057 | 0·0067 | ... |
| 9-10 ,, | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| 10-11 ,, | 0·059$^p$ | ... | ... | 0·067 | ... | ... | ... | 0·0029 | 0·0059 | ... |
| 11-12 ,, | ... | ... | ... | ... | 0·077 | ... | 0·0022 | ... | 0·0050 | ... |
| 24 hrs. | 0·045$^p$ | 0·16$^{pp}$ | 0·070 | 0·064 | 0·077 | 0·030 | 0·00095 | ... | 0·0011 | ... |
| 30 ,, | 0·050$^p$ | 0·13$^{pppp}$ | 0·057 | 0·056 | ... | ... | ... | ... | ... | ... |
| 48 ,, | 0·010$^p$ | $^{pppp}$ | 0·034 | ... | ... | ... | ... | ... | ... | ... |
| 54 ,, | 0·022$^p$ | ... | ... | ... | 0·057 | 0·0087 | ... | ... | ... | ... |
| 72 ,, | 0·026$^p$ | 0·039$^{pp}$ | ... | 0·030 | ... | ... | ... | ... | ... | ... |
| 78 ,, | ... | ... | ... | 0·019 | ... | ... | ... | ... | ... | ... |
| 96 ,, | 0·023$^p$ | ... | ... | 0·018 | ... | ... | ... | ... | ... | ... |
| 120 ,, | ... | ... | 0·0057 | ... | ... | ... | ... | ... | ... | ... |

$^p$ = 50 milli units posterior lobe of the pituitary extract given before milking.

*No.* 235. Milking started 5 days after delivery when litter died. Machine used.

*No.* 237. Milking started 10 days after delivery. Machine used. Two sucklings left. Rab died suddenly.

*No.* 258. Milking started by hand 2 days after delivery—3 sucklings.

*No.* 232. Milking started by hand 3 days after delivery—2 sucklings.

*No.* 257. Milking started by hand 4 days after delivery—sucklings died.

*No.* 284. Milking started by hand 8 days after delivery—1 suckling.

Great difficulty was experienced in obtaining any milk with the machine; injections of extract of posterior lobe of the pituitary were given to three rabbits. Hand milking proved far more satisfactory and after its introduction no further pituitary extract was needed.

A high percentage of the injected strontium appeared in the milk in rabbit number 237 (table VI; fig. 2) which received pituitary extract, the maximum of 0·41 per cent. being found 3-4 hours after injection. An excretion of 0·21 per cent. was found in rabbit number 258 7-8 hours after injection, a single dose only of pituitary extract having been given in this case. In the other 4 rabbits a much lower excretion of strontium was noted, the peak occurring within 8 hours of injection. By the third day the level was never greater than 0·04 per cent. and by the fifth day had fallen to 0·0057 per cent. The percentage of strontium in the blood following injection fell rapidly both in lactating and non-lactating rabbits (fig. 3). Five minutes after injection 1 ml. of blood contained 0·17 per cent. of the injected dose, after one hour it had fallen to 0·04 per cent. and after 4 hours to 0·01 per cent. Twelve hours after the injection it was 0·002 per cent.

EXCRETION OF STRONTIUM IN THE MILK OF RABBITS FOLLOWING INTRAVENOUS INJECTION

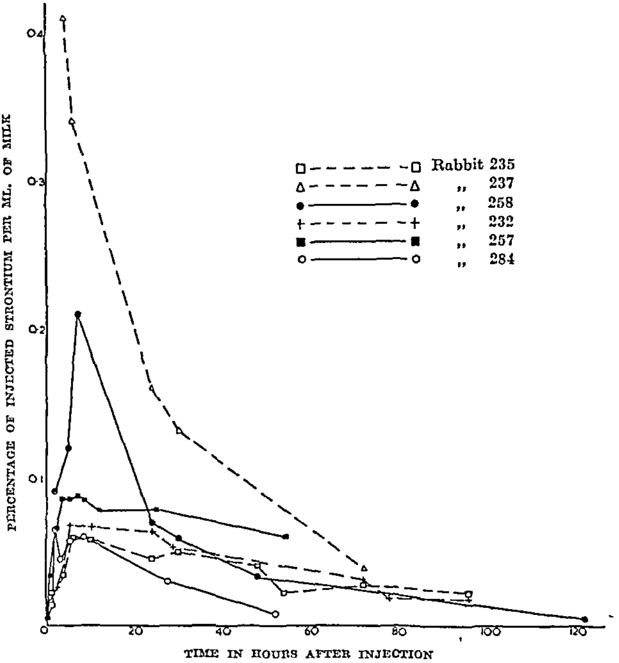

FIG. 2.—The level of radioactive strontium in the milk following intravenous injection of a single dose.

### Excretion of injected strontium in the milk

### (a) *Mother injected* 38 *days before delivery*

The activity of a sample of milk withdrawn two to three days after delivery from two rabbits injected 38 days before delivery was so slight as to be only just above background and no accurate estimate of the strontium content could be made. The activity of the blood withdrawn from rabbits 50 days after injection was so low that calculation of the strontium content was not justified.

### (b) *Mother injected* 2-10 *days after delivery*

In rabbits numbers 235 and 257 the litters died before milking began. The other four rabbits were returned to their litters from time to time.

TABLE VI

*Percentage of injected strontium in milk and blood of individual rabbits*

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|---|---|---|---|---|---|---|---|---|---|---|
| | 235 | 237 | 258 | 232 | 257 | 284 | 257 | 284 | 73 | 68 |
| 5 minutes | ... | ... | ... | ... | ... | ... | 0·17 | ... | ... | ... |
| 10 ,, | ... | ... | ... | ... | 0·0053 | ... | ... | 0·11 | 0·16 | 0·19 |
| 10 mins.-1 hr. | ... | ... | ... | 0·016 | ... | 0·0052 | ... | ... | ... | ... |
| 1-2 hrs. | 0·021$^{p}$ | ... | ... | ... | 0·035 | 0·014 | 0·044 ... | 0·015 | ... | ... |
| 2-3 ,, | ... | ... | 0·091$^{p}$ | 0·026 | 0·067 | 0·068 | 0·039 | 0·029 | 0·058 | 0·045 |
| 3-4 ,, | ... | ... | ... | ... | 0·085 | 0·017 | 0·036 | 0·025 | ... | ... |
| 4-5 ,, | 0·033 | 0·41$^{pppp}$ | ... | 0·041 | 0·085 | ... | 0·014 | ... | 0·0095 | 0·01 |
| 5-6 ,, | ... | ... | 0·12 | 0·068 | ... | 0·050 | ... | 0·011 | ... | 0·009 |
| 6-7 ,, | 0·060$^{p}$ | 0·30$^{p}$ | ... | ... | ... | ... | 0·0087 | ... | ... | ... |
| 7-8 ,, | ... | ... | 0·21 | ... | 0·087 | ... | ... | ... | ... | ... |
| 8-9 ,, | ... | ... | ... | ... | 0·086 | 0·062 | 0·0067 | 0·0057 | 0·0067 | ... |
| 9-10 ,, | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| 10-11 ,, | 0·059$^{p}$ | ... | ... | 0·067 | ... | ... | ... | 0·0029 | 0·0059 | ... |
| 11-12 ,, | ... | ... | ... | ... | 0·077 | ... | 0·0022 | ... | 0·0050 | ... |
| 24 hrs. | 0·045$^{p}$ | 0·16$^{pp}$ | 0·070 | 0·064 | 0·077 | 0·030 | 0·00095 | ... | 0·0011 | ... |
| 30 ,, | 0·050$^{p}$ | 0·13$^{pppp}$ | 0·057 | 0·056 | ... | ... | ... | ... | ... | ... |
| 48 ,, | 0·040$^{p}$ | $^{pppp}$ | 0·034 | ... | ... | ... | ... | ... | ... | ... |
| 54 ,, | 0·022$^{p}$ | ... | ... | ... | 0·057 | 0·0087 | ... | ... | ... | ... |
| 72 ,, | 0·026$^{p}$ | 0·039$^{pp}$ | ... | 0·030 | ... | ... | ... | ... | ... | ... |
| 78 ,, | ... | ... | ... | 0·019 | ... | ... | ... | ... | ... | ... |
| 96 ,, | 0·023$^{p}$ | ... | ... | 0·018 | ... | ... | ... | | ... | ... |
| 120 ,, | ... | ... | 0·0057 | ... | ... | ... | ... | ... | ... | ... |

$^{p}$ = 50 milli units posterior lobe of the pituitary extract given before milking.

*No.* 235. Milking started 5 days after delivery when litter died. Machine used.

*No.* 237. Milking started 10 days after delivery. Machine used. Two sucklings left. Rabb died suddenly.

*No.* 258. Milking started by hand 2 days after delivery—3 sucklings.

*No.* 232. Milking started by hand 3 days after delivery—2 sucklings.

*No. 257. Milking started by hand 4 days after delivery—sucklings died.*

*No.* 284. Milking started by hand 8 days after delivery—1 suckling.

Great difficulty was experienced in obtaining any milk with the machine; injections of extract of posterior lobe of the pituitary were given to three rabbits. Hand milking proved far more satisfactory and after its introduction no further pituitary extract was needed.

### (b) *Rabbits injected* 9 *days before delivery*

In rabbits injected 9 days before delivery, $Y^{90}$ present from injection, 9 days previously, can be neglected at the time of death. In the mother's bones and in the fœtuses, $Sr^{90}$ and $Y^{90}$ were very approximately in equilibrium.

### (c) *Rabbits injected* 24 *hours before delivery*

In rabbits injected 24 hours before delivery, $Y^{90}$ in the fœtuses as well as in the mothers' bones was very approximately in equilibrium with $Sr^{90}$. Decay curves done on two placentas showed a concentration of $Y^{90}$ greater than that of $Sr^{90}$.

### (d) *Lactating and Suckling rabbits*

The bones were not in complete radioactive equilibrium when removed from the body. The differences observed were, however, too slight to allow of accurate calculation of the yttrium content. The figures suggest that the percentage of yttrium present is approximately the same as that of strontium.

### (e) *Blood*

Samples of blood were not in radioactive equilibrium when withdrawn. It has not proved possible to calculate the yttrium content by the decay curves method (Kidman *et al.*) as A.E.R.E., Harwell has been unable to give figures for the relative proportion of $Sr^{89}$ and $Sr^{90}$ in the particular solution used in this experiment. The maximum differences between the initial count and that obtained after 20 days when equilibrium was established was not more than 20-30 per cent.

## DISCUSSION

As in adults and weanlings the retention of strontium by pregnant and lactating rabbits and their offspring is affected by diet, being greater in a rabbit on a diet low in calcium than one high in calcium.

Radioactive strontium passes through the placenta to the fœtus in rabbits as it does in mice (Finkel, 1947), and through the milk. The source of this strontium is probably twofold, (i) from the strontium stored in the maternal bones, (ii) from the injected strontium circulating in the blood in the case of animals injected late in pregnancy or during lactation.

The fact that the mean retention in non-pregnant six-months-old rabbits on a medium calcium diet is 17·1 per cent. while that of pregnant rabbits is 12·7 per cent., suggests that some may pass from the bones to the fœtus. The amount transferred from the maternal bones is, however, small. The mean figure for the strontium content of the bones of the mother on a medium calcium diet and injected 9 days before delivery was 12·7 per cent. while the mean figure for the bones of mothers injected 38 days before delivery was approximately the same, 12·1 per cent. The mothers in the latter group had clearly lost little to their litters as is proved by analysis of the strontium content of the litters. In the case of mothers injected 9 days before delivery the litters' content of strontium was 0·8 per cent. while in the litters of mothers injected 38 days before delivery it was 0·03 per cent. These figures suggest that pregnancy does not lead to any dramatic mobilisation of strontium from the skeleton. Strontium given to the mother late in pregnancy passes to the fœtus presumably from that circulating in the blood immediately following injection. There is a rapid fall from a level of approximately 17 per cent. per 100 ml. 5 minutes after injection to 4·4 per cent., per 100 ml. at the end of 1 hour and 0·1 per cent. per 100 ml. at 24 hours. Forty-four days after injection the circulating blood contained only a trace of radioactive strontium which could not be measured accurately.

The amount retained by the fœtus is greater the later in pregnancy the injection is given. The mean figure for retention per fœtus when the mother was injected 9 days before delivery was *only* 0·2 per cent. compared with 1·5 per cent. when injection of the mother occurred 24 hours before delivery. Observations on the rabbit here again agree with those on the mouse (Finkel). This is presumably associated with the activity of calcification. Calcification is recorded as being found on section of the embryo on the 16th day (Minot and Taylor, 1905; Brambell, 1949, personal communication). It is present as shown by X-ray examination 11 days before delivery, *i.e.* on the 20th day: it is much more advanced 24 hours before delivery. It is not possible to detect it by X-ray technique on the 18th day (fig. 4). It is not surprising therefore that if an injection is given 24 hours before delivery the retention of strontium is greater than if it is given 9 days before delivery. There is in fact actual concentration in fœtal tissue when the mothers are injected 24 hours before delivery (table III). The maximum concentration in the blood in this case corresponds with a higher demand on the part of the bones for minerals. In rabbits injected late in pregnancy it is probable that the greater part of the strontium in the fœtuses has come from the blood immediately following the injection, but some has possibly also been drawn from the mother's bones since these contain less than those of virgin rabbits of approximately the same age and on the same diet.

The numbers in the present experiments are small but the retention in litter and mother together appears, with the exception of the high calcium group, to be rather less than in the non-pregnant animal. This is particularly noticeable in the low calcium group, differing slightly from the observations of Finkel on mice. She considered that the amount of $Sr^{89}$ retained immediately after delivery was the same as that retained by virgin adult mice at a comparable time interval after injection. She suggested that a portion of the material that would have been excreted was lost through the placenta. The observations here suggest that some of the strontium that would have been in the mother's bones had passed to the litter through the placenta.

It may be objected that in many cases the fœtuses of mothers injected 24 hours before delivery had milk in their stomachs and had therefore absorbed some of the strontium in their bones from this source. This is theoretically correct, though it is perhaps doubtful whether much could be gained. Milk found in the fœtal stomach had a maximum strontium content of 0·6 per cent. and in one group the amount retained per fœtus in two litters was not greater in the fœtuses that had milk in the stomach than in the fœtuses of one litter that had not. It appears reasonable to conclude that, irrespective of the strontium in the milk that was available to some litters, more strontium was retained by the fœtus when the mother was injected late in pregnancy.

The total amount retained per litter appears, in the small number of results recorded, to be affected by the size of the litter, being greater the bigger the litter. The amount retained per fœtus is affected by the diet of the mother. The fœtus of the mother on a low calcium diet retains six times as much as the fœtus of the mother on a medium or high calcium diet when the injection is given 9 days before delivery and three times as much when the injection is given 24 hours before delivery.

There is evidence that yttrium as well as strontium passes across the placenta. The fact that $Y^{90}$ was approximately in equilibrium with $Sr^{90}$ in the bones of fœtuses whose mothers were injected 24 hours before delivery shows that $Y^{90}$ must have passed through the placenta, since even assuming that all the $Sr^{90}$ in these fœtuses was deposited within an hour of injection only 50 per cent. of the equilibrium concentration could have been restored. There is no striking differential excretion of $Y^{90}$ since $Sr^{90}$ and $Y^{90}$ were in approximate equilibrium in all the fœtuses examined: $Y^{90}$ was therefore as dangerous to these young animals as to the older animals already studied.

It is not easy to interpret the effect of diet on retention of strontium in lactating rabbits and their offspring owing to the presence of factors which were not present in the animals previously studied. For instance, the lactating mothers and their sucklings on a low calcium diet had a sudden and uncontrolled additional source of calcium available from the hay of the nest during the period of lactation. Further the strontium found in the sucklings may have come in part from that injected into the blood stream and reaching the milk directly and in part from that subsequently liberated into the blood stream from the bones. Some of these complications might be analysed by further experiments designed for this purpose but it is doubtful whether the additional clarification would justify the labour involved.

It is clear that retention of strontium by the suckling and lactating rabbit is affected by the diet of the mother when the strontium is injected during lactation. The lactating rabbit on a low calcium diet and nested on straw which is poor in calcium retained twice as much as when nested on hay and five times as much as the rabbit on a high calcium intake. An increased retention in the low calcium sucklings per g. of bone was also apparent. Suckling rabbits as a whole retained considerably more than their mothers. This is remarkable when it is remembered that in the case of sucklings the strontium was absorbed from the alimentary canal. Absorption of strontium from the stomach and intestine is not so efficient as absorption from the blood stream though evidence about the degree of intestinal efficiency is conflicting. Hamilton (1948) in early work with adult rats reports that 15 per cent. or less of orally administered radio-strontium was absorbed through the gastro-intestinal tract. Later he states that in rats 5-60 per cent. of the dose administered by mouth is absorbed without giving information about age and diet (Hamilton, 1947). Copp *et al.* (1947) also report a high retention of the order of 70 per cent. following oral administration to young rats on a low calcium diet. Scott *et al.* (1947) on the other hand state that in rats only about 15 per cent. of orally administered radio-strontium is absorbed. The effect of age of the mother at the time of lactation on retention in the suckling was not investigated in detail—a few scattered observations suggest that it was insignificant. Lactating mothers injected 7 days after delivery irrespective of their diets had a smaller retention in their bones than virgin rabbits. This was most noticeable in rabbits on a low calcium diet and may be in part explained by the fact that lactating rabbits on such a diet had access to hay while their virgin controls had not. The retention of strontium in virgin rabbits on a low calcium intake given access to hay for a comparable period was also much less than in those which had no hay. The fact that the lactating mothers were two months older than the controls should not alone account for this difference. An 18-months-old rabbit retained more than an 8-months-old lactating rabbit. The virgin 6-7-months-old rabbit on a medium calcium diet retained an average of 17·1 per cent. in its bones while the lactating 8-months-old rabbit, injected after delivery, retained an average of 6·6 per cent. (Kidman *et al.*). This difference suggests that strontium was to some small extent withdrawn from the bones into the milk when the strontium was given after delivery, and had only recently been taken up by the bone. On the other hand the young of rabbits injected before pregnancy contain only traces of strontium and fostered sucklings nursed by mothers injected before pregnancy also contain little strontium, suggesting that strontium which has been long in the bone is mobilised slowly. The form in which strontium is present in the bone at different times following injection requires further study.

It is difficult to compare the present figures for strontium excretion into the milk with those obtained by Erf and Pecher (1940) in cows.

The cows were completely stripped of milk at each milking and the total strontium excretion could therefore be estimated. It was impossible to strip the rabbits at each milking and only one (no. 232) had a litter that survived the whole experiment. The results can only be given per ml. of milk and the milk expressed in any sample may have been long in the mammary tissue. The shape of the curves of excretion of strontium in the milk are variable, but in all there appears to be a considerable flattening of the curve after the first day. The strontium in the later samples may have come from that secreted in the first few hours into the milk which was then stored in the mammary tissue. It is noticeable, however, that similar results were obtained from the milk of rabbit no. 232 which retained her litter, and who may have been stripped, as from the milk of mothers whose litters died. In two animals there was an appreciable peak of excretion, in the other three this was much less noticeable. The two animals with a peak both received pituitary extract, but a third animal (no. 235) who received large doses of extract showed a low excretion. Owing to the many variables present, too much stress should not be laid upon the absolute values of excretion obtained. It can be concluded only that strontium is excreted into the milk following injection into the blood stream after delivery, a maximum excretion being reached within 8 hours. There is clearly a considerable risk of the passage of radioactive strontium to the offspring of lactating rabbits from the intake of a single dose by the mother during lactation. The risks of mobilisation and transmission of strontium laid down in small amounts in the bones before pregnancy are less, though the effects of such chronic poisoning on fertility and survival of the litter cannot be overlooked (Finkel).

## SUMMARY

1. Experiments are described on the retention of strontium and yttrium in pregnant and lactating rabbits and their offspring.

2. Radioactive strontium injected into the pregnant rabbit before delivery passes through the placenta to the litter, the amount retained being related to the time of injection, the diet of the mother and the size of the litter.

3. The strontium in the fœtus is derived to a small extent from that stored in the maternal tissues. Pregnancy does not result in any dramatic mobilisation of strontium from the mother's bones. If the mother is injected late in pregnancy appreciable amounts of the injected strontium circulating in the maternal blood are taken up by the actively calcifying fœtal skeleton.

4. The pregnant rabbit on a low calcium diet retains less than the virgin rabbit at a comparable period after injection. On a high calcium diet the difference is insignificant.

5. The lactating rabbit retains slightly less strontium and $Y^{90}$ produced by the decay of $Sr^{90}$ than the pregnant or virgin rabbit

of the same age and in the same diet group. The lactating rabbit on a low calcium intake retains approximately twice as much as the rabbits on a medium and high calcium diet.

6. Strontium injected into the lactating rabbit passes through the milk to the suckling. Sucklings of mothers on a low calcium diet retain more than those on a high or medium calcium diet.

7. Strontium stored in the bones before pregnancy is mobilised only in insignificant amounts during lactation.

8. The peak of excretion of strontium in the milk following an intravenous injection occurs when the level in the blood is low.

9. The retention of yttrium appears to be roughly proportional to that of strontium in pregnant and lactating rabbits and their offspring.

We should like to express our thanks to Professor J. H. Burn, whose constant help and encouragement has made this work possible, and to many members of the staff at A.E.R.E., Harwell. We are also grateful to Dr Henry for the use of her electric milking machine. One of us (J. V.) has an expenses grant from the Medical Research Council. We are also indebted to the Hæmatological Research Fund endowed by the Nuffield Foundation.

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# A HISTOCHEMICAL AND BIOCHEMICAL STUDY OF SOME EFFECTS PRODUCED BY THE ROUS SARCOMA UPON THE ENDOCRINE ORGANS OF COCKS AND HENS

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(PLATES XLIII-XLVI)

THE isolation from the urine of cancer patients of abnormal steroids (Butler and Marrian, 1937, 1938; Dobriner *et al.*, 1944) suggests the possibility that tumour tissue might produce by-products of a steroid nature which would affect the functions of the endocrine organs. The steroid-producing organs are easily influenced by injected steroids and show great physiological interdependence. It therefore appeared logical to examine them for changes in the tumour-bearing animals. Some work along these lines has already been done. Ball and Samuels (1938) found evidence of adrenal hypertrophy in tumour-bearing rats: this was prevented by hypophysectomy. Dalton and Peters (1944-45) found lipoid depletion of the adrenal cortex in mice with transplantable carcinomata, leukæmia Y 103 and sarcomata. It was noted that in the case of the transplantable sarcomata, the loss of lipid from the adrenal showed no correlation with the degree of necrosis of the tumour, which suggested that the mere presence of a large mass of actively growing tumour tissue might be capable of stimulating the release of cortical lipids. This effect was more easily seen in male mice because they had less cortical lipid than the females. Dalton and Peters used several different tumours but concentrated their study upon only one endocrine organ. I have used only one tumour but have studied several of the sterol-producing organs in an attempt to get a more complete endocrine picture.

The earliest experiments in the present investigation were made by transplanting tumours on to the chorio-allantois of embryo chicks, a technique which has the advantage that a number of different tumours, including those from different species, can be grown. Rous sarcoma, Jensen rat sarcoma and a seminoma from a human patient were all successfully grown. It was also argued that the chick embryo with its full complement of immature, labile and rapidly developing endocrine organs should form good biological material for steroid analysis. This material, however, suffers from two great disadvantages, of the same age and in the same diet group. The lactating rabbit on a low calcium intake retains approximately twice as much as the rabbits on a medium and high calcium diet.

6. Strontium injected into the lactating rabbit passes through the milk to the suckling. Sucklings of mothers on a low calcium diet retain more than those on a high or medium calcium diet.

7. Strontium stored in the bones before pregnancy is mobilised only in insignificant amounts during lactation.

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feeding, etc. The birds came to autopsy 3 weeks after injection, when they had nearly always grown large tumours, but before marked signs of constitutional disturbance had set in. We were anxious to avoid changes which might appear merely in moribund birds.

The birds were killed by cervical dislocation and the ovaries, testes, adrenals and preen glands were removed immediately after death. Each organ was divided and one part placed in a test-tube cooled in a freezing mixture and then kept in the −20° C. refrigerator until it could be analysed for ascorbic-acid content. The other part was frozen on a plate cooled with $CO_2$ snow and cut into slices 1·2 mm. thick which were placed in the following fixatives for histochemical examination.

*For ascorbic acid,* the method of Barnet and Bourne (1941-42) was used. The tissues were fixed in 10 per cent. silver nitrate-10 per cent. acetic acid mixture for 3 hrs., washed in three changes of distilled water (1½ hrs.) and 5 per cent. sodium thiosulphate (1½ hrs.). Chemically clean glassware and analar reagents were used and the process was carried out in the dark. The material was dehydrated, cleared, embedded in paraffin, cut at 5 $\mu$ and lightly counterstained with neutral red. It was found that the longer time (3 hrs.) in silver-acetic fixative gave fewer artefacts, silver tides, etc., than the shorter half-hour period of fixation suggested in a personal communication by Dr G. Bourne.

*For lipids,* the method of Dalton and Peters was used. The tissues were fixed in Champy's fluid for 24 hrs., washed in running tap water for 24 hrs. and then placed in 2 per cent. osmic acid for 5 days at 38° C. Following embedding in paraffin, sections were cut at 5 $\mu$ and either mounted unstained or lightly counterstained with neutral red.

*For alkaline phosphatase,* the Gomori technique was used, keeping within the time limits, etc., suggested by Danielli (1946). The tissues were fixed in 80 per cent. ice-cold ethanol for 24 hrs. The sections were incubated in the substrate for 2, 6 and 24 hrs., and compared with controls incubated at *p*H 9 without $\beta$ glycero-phosphate for identical periods of time. Tissues from the tumour-bearing birds and from the controls were incubated back to back.

*For acid phosphatase,* the Gomori technique (1941) was used. The tissues were fixed in ice-cold absolute acetone for 24 hrs. The sections were incubated in an acetate buffer at *p*H 5 with $\alpha$ glycero-phosphate and lead nitrate for 2, 6 and 24 hrs. and compared with the controls.

*For polysaccharide,* the periodic acid-Schiff's reagent (P.A.S.) method (McManus, 1948) was used. This stains the 1, 2 glycol linkage (−CHOH−CHOH−) of carbohydrates and thus stains glycogen, mucus, reticulin, basement membranes, some kinds of elastic tissue, fibrin and various substances of quite unknown composition. The tissues were fixed in 80 per cent. alcohol.

*For the pituitary,* the method of Dawson and Friedgood (1938) was used. The pituitaries were dissected out and fixed either in the silver-acetic mixture for ascorbic acid or in formol-saline-sublimate. The method makes use of azo-carmine, orange G and aniline blue, and it was found that it would distinguish acidophils and basophils even in pituitaries which had been fixed in silver-acetic. The appearance of the cells and the overall intensity of staining differ with the [illegible] extracts of the various tissues were carried out by means of 2 : 6 dichlorophenol indophenol titration.

## RESULTS

### *Biochemical estimation of ascorbic acid content*

Fig. 1 shows that the ascorbic-acid content of the adrenals is very variable and that there is no significant difference between the normal birds and the tumour-bearing cocks or hens. There is no suggestion that the ascorbic-acid content of the testes is decreased in the tumour-bearing cocks in spite of the marked degenerative changes seen on histological examination. The small number of results available, however, show no statistically significant increase. The ascorbic-acid content of the ovaries of the tumour-bearing birds shows a small but significant increase. In this case, however, there is little histological change from normal and certainly no marked degenerative changes such as are found in the testes.

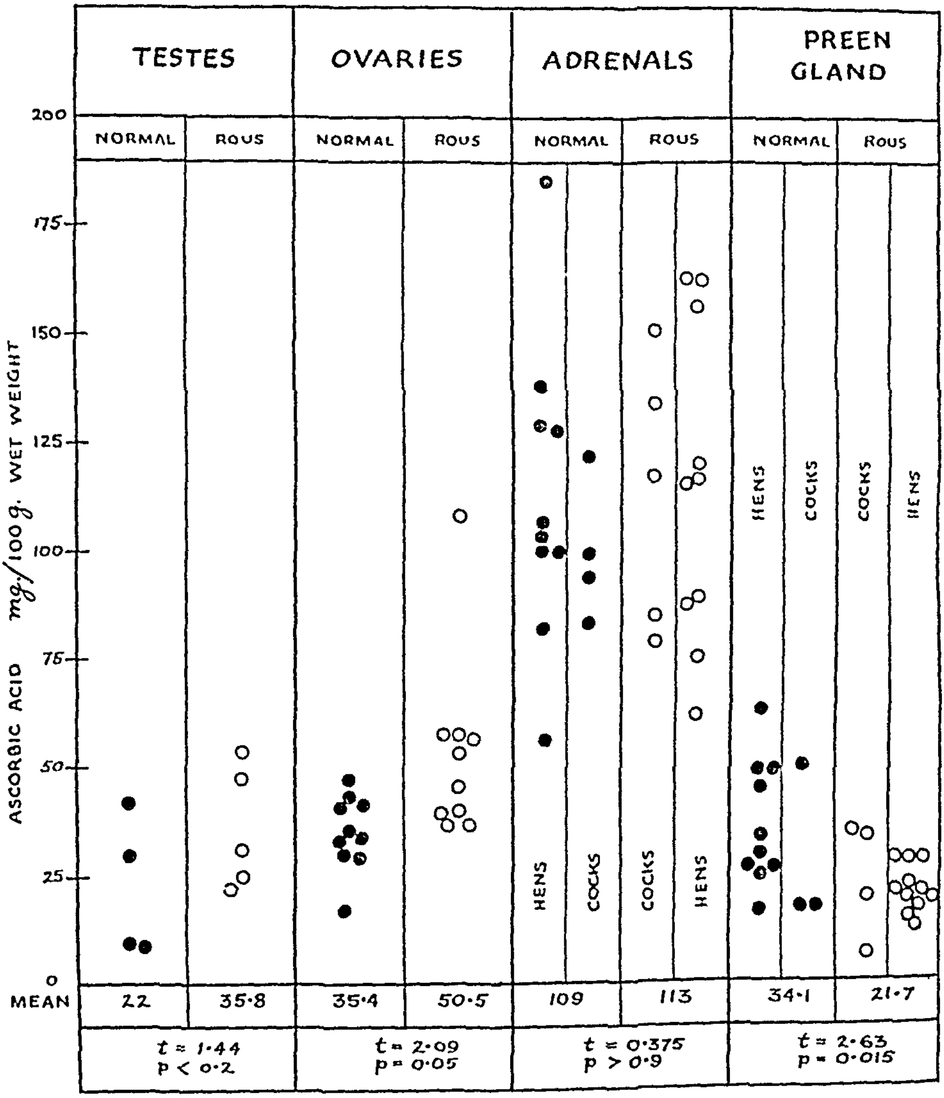

Fig. 1.—Biochemical estimation of ascorbic-acid content.

The value of the ascorbic-acid content of the preen glands varies between 16 and 49 mg./100 g. wet weight (average 34·1) for normal birds and between 7 and 35 mg. (average 21·7) for the Rous-infected cocks and hens. It was also noticed that the preen glands of the tumour-bearing birds are considerably smaller than the normal controls ($P<0·001$) and that this diminution in weight is greater than can be accounted for by any loss of body-weight. It was noticed also that the preen glands of the Rous birds contain more secretion than the controls. A relative increase in the quantity of secretion to the amount of gland parenchyma will lower the ascorbic-acid wet-weight figure, but if a gland is enlarged by secretion without diminution of glandular tissue the product of the weight of the gland × ascorbic acid/100 g. wet weight will remain constant if the secretion has a negligible ascorbic-acid content. If ascorbic acid had any relationship to gland function the total ascorbic-acid content of the whole organ per gram of body-weight might be more significant than the concentration in the organ.

Fig. 2 shows the ascorbic acid in the whole organ per gramme of body-weight for both the preen gland and the adrenals. These two organs are in sharp contrast. In the case of the adrenals, fig. 2 shows less scatter than fig. 1. In the case of the preen gland the differences between the normal and the Rous birds are more marked. This indicates that in the adrenals the larger glands tend to have a lower concentration of ascorbic acid so that the total adrenal ascorbic acid per gramme of whole bird tends to be constant, with no significant difference between the tumour-bearing birds and the controls. In the preen gland there is either a loss of weight or a lower concentration of ascorbic acid or both in the Rous birds, which is a finding consistent with the marked degenerative changes seen on histological examination.

*Ascorbic-acid content of the Rous sarcoma.* Tumours usually give low values of ascorbic acid—3 or 4 mg./100 g. wet weight. These low values may be explained by the large quantity of extracellular ground substance which is such a characteristic feature of the Rous sarcoma. One small tumour, only 4·5 per cent. of body-weight and more cellular than usual, gave a value of 10 mg./100 g. wet weight. Rous sarcoma cells grown on the chorio-allantois of the duck embryo, and therefore free from muscle, gave 5 or 6 mg./100 g. wet weight. These low values are interesting in comparison with the high values of ascorbic acid in most other malignant tumours.

*Naked-eye appearance of tumour.* The larger Rous tumours of the series showed the typical gelatinous consistency. The centre had usually broken down to form a cyst which was filled with hæmorrhagic fluid and lined by fungating masses of growth. Every bird in which the tumour was at all extensive showed wide-spread subcutaneous gelatinous exudate, usually bright green in colour. Small tumours showed a firmer, whiter and less gelatinous structure.

*Metastatic deposits.* Metastases visible to the naked eye were infrequent. One bird showed multiple deposits in the liver; three showed metastatic deposits in the preen gland. This is interesting because the ovaries, testes and adrenal glands were also submitted to close histological scrutiny without finding any secondary deposits.

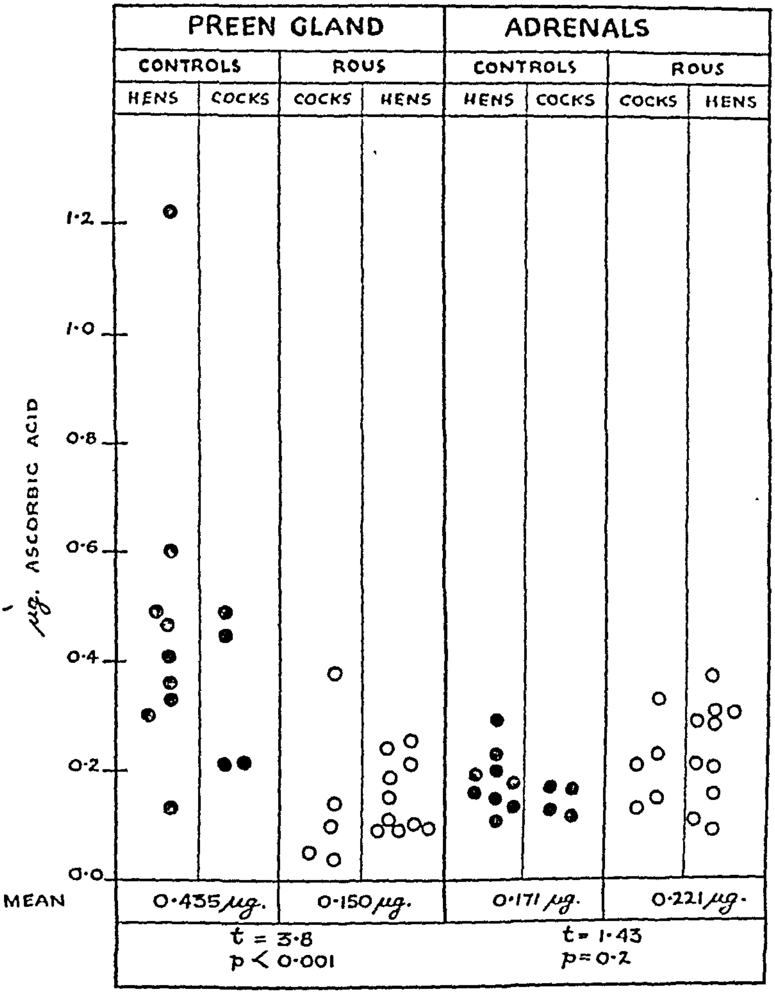

FIG. 2.—μg. ascorbic acid in whole preen gland and both adrenals per gramme of body-weight.

Recently, two more birds with secondary deposits in the preen gland have been found among four birds used for class work.

### *Rous sarcoma: histology and histochemistry*

*Ascorbic acid.* Silver-reacting granules, indicating ascorbic acid in the histochemical technique used, are very scanty in the Rous sarcoma. This is in keeping with the low ascorbic-acid content found on biochemical analysis. Most of the Rous sarcoma cells are free from silver-reacting granules, an occasional cell shows a fine granule in the cytoplasm adjacent to the nucleus and in some preparations a few groups of cells are found with several silver-reacting granules.

## ROUS SARCOMA AND ENDOCRINE ORGANS

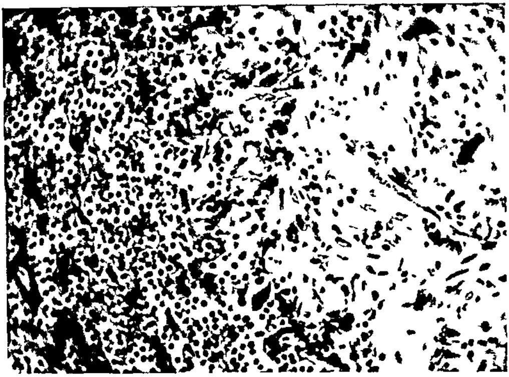

FIG 3—Secondary deposit of Rous sarcoma (right) in liver (left) Gomori technique for acid phosphatase, 24 hrs incubation Extracellular matrix and cytoplasm of Rous sarcoma cells are unstained and the nuclei stain only moderately In contrast, the nuclei of the liver cells stain strongly and the nuclei and cytoplasm of the Kupffer cells very strongly ×95

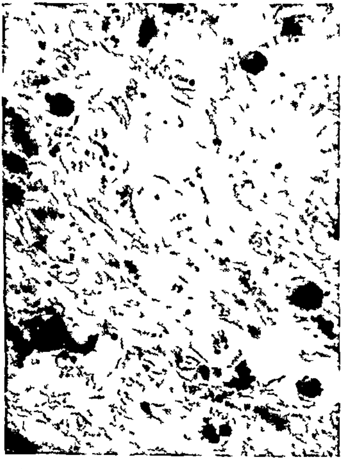

FIG 4—Secondary deposit of Rous sarcoma in liver A zone of active mitosis showing lipoid deposits Osmic acid and neutral red ×400

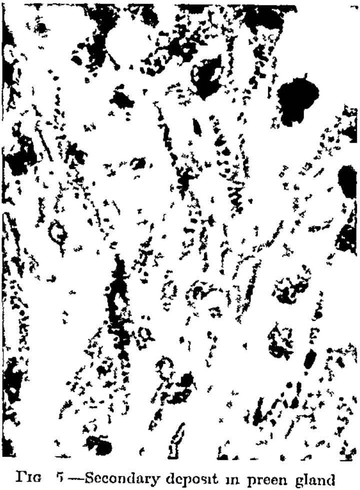

FIG 5—Secondary deposit in preen gland showing P A S positive granules in the Rous sarcoma cells ×400

*Alkaline phosphatase.* With the possible exception of the nucleoli, the Rous sarcoma shows no staining for alkaline phosphatase even after 24 hrs.' incubation. It is interesting to find this combination of low ascorbic-acid content and absence of extracellular alkaline phosphatase in a tumour which makes much hyaluronic acid but no collagen.

*Acid phosphatase.* The nuclear chromatin of the sarcoma cells gives a positive reaction for acid phosphatase after 24 hrs.' incubation but the cytoplasm and the extracellular matrix are free. The cells lining the capillaries in the tumour stain positively.

A study of the secondary deposits of the Rous sarcoma in the liver and preen gland is instructive. With the naked eye the tumour can be seen as a pale zone against the darkly staining background of the liver and preen gland. Some idea of intensity of phosphatase activity can be gained by comparing sections incubated for 2, 6 and 24 hrs. Intense phosphatase activity would be indicated by complete blackening within 2 hrs., whereas a low degree of activity would give perceptible staining only after 24 hrs.' incubation.

The results from the secondary deposits of Rous sarcoma in the liver (fig. 3) are shown in the table. Similar results were given by the primary tumours and by the secondary deposits in the preen gland.

TABLE

*Acid-phosphatase activity of liver and Rous sarcoma*

| | Period of incubation | | |
|---|---|---|---|
| | 2 hrs | 6 hrs | 24 hrs |
| LIVER | | | |
| Liver-cell nuclei | – | ± | ++ |
| Liver-cell cytoplasm | – | – | + |
| Kupffer cells | – | ++ | ++++ |
| ROUS SARCOMA | | | |
| Cytoplasm and matrix | – | – | – |
| Tumour-cell nuclei | – | – | + |
| Chromatin of cells in mitosis | – | – | ++ |
| Polymorphonuclear leucocytes | – | – | ++ |

*Lipid.* Many Rous sarcoma cells show no lipoid droplets stainable with osmic acid after 4 days' incubation, but groups of cells can be found which contain collections of fine or coarse lipoid droplets. At first these were regarded as degenerative changes but further study showed that lipoid droplets occurred in actively growing parts of the tumour. Fig. 4, from a secondary in the liver, shows 3 mitotic figures, and two of these dividing cells each possess a dark granule while several other cells in the field show considerable collections of lipid. Other parts of the same tumour present typical spindle cells containing rows of black droplets.

*Polysaccharide.* With the P.A.S. staining technique the extracellular matrix of the tumour stains diffusely or with fine bands. Some groups of tumour cells contain collections of brightly staining granules of polysaccharide in their cytoplasm. Fig. 5, for example, shows actively growing cells from a metastatic deposit in the preen gland, and there is no suggestion that these cells are in any way degenerate. Are these cytoplasmic granules of polysaccharide glycogen ? To answer this question, a careful study was made of the secondary deposits in the liver. Incubation with salivary amylase for ¼ and ½ hr. removed the glycogen granules from the liver cells but the number of P.A.S.-positve granules in the Rous sarcoma was comparable with those in control sections incubated for similar periods in boiled saliva. Whether these polysaccharide granules have any relationship with the lipoid droplets or whether they represent the precursor in the cells of the extracellular matrix can only be answered by further investigation.

### *Testes : histology and histochemistry*

The testes in the tumour-bearing cocks showed marked degenerative changes. They were smaller than in the two normal controls, and yellow instead of white.

Figs. 6 and 7 show the striking histological differences between the testis from a tumour-bearing cock and an immature testis of the same order of size from a normal cock. The testis cords of the normal bird show three or four layers of cells and a very narrow lumen. In the tumour bird the cords are changed into wide tubules, lined in places by only a single layer of cells and containing plugs of desquamated cells lying free in the enlarged lumen. These free cells have deeply-staining pyknotic nuclei and the basement membrane of the tubules is thickened, a feature which is shown best in sections stained with P.A.S.

With silver-acetic acid fixation silver-reacting granules are well marked in these degenerate tubules in the cell borders next to the lumen (*cf.* figs. 8 and 9). Osmic acid stains lipoid droplets in the same situation (fig. 10).

With P.A.S. staining many fine granules of polysaccharide are seen in this situation and the desquamated cells are also P.A.S. positive, showing a ring of intensely staining granules surrounding the nucleus. These remain unaltered even after incubation with salivary amylase for 2 hrs. (fig. 11).

The interstitial cells of the testes are significantly more numerous in the tumour-bearing cocks but this may be the result of retardation of development. They do not appear to stain so well, either for lipid or for ascorbic acid, in the Rous-infected birds as in the controls.

*Phosphatases in the testes.* The testes give a positive reaction for both acid and alkaline phosphatase but the distribution of each is characteristic and different. Alkaline phosphatase is weakly positive

ROUS SARCOMA AND ENDOCRINE ORGANS

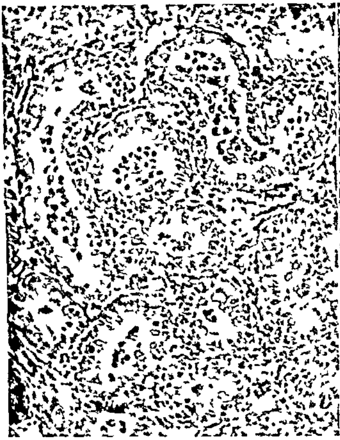

FIG 6 —Testis from tumour bearing cock The tubules are one cell thick and there are many cells in the lumen Hæmatoxylin and eosin ×200

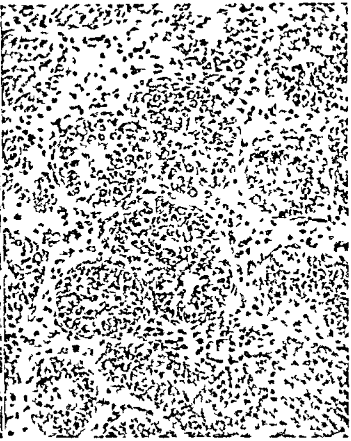

FIG 7 —Normal testis The tubules are 3 or 4 cells thick and the lumen is small H and E ×200

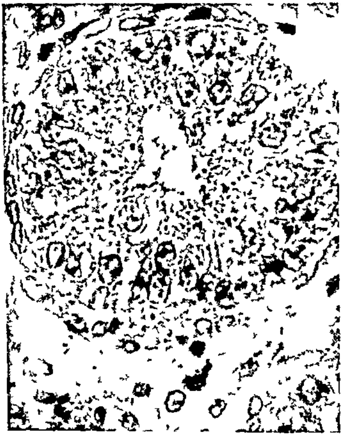

FIG 8 —Testis of normal cock Silver acetic and neutral red Ascorbic acid shown as tiny granules near lumen of tubule Wt of testis 480 mg Ascorbic acid 29 mg /100 g wet wt ×945

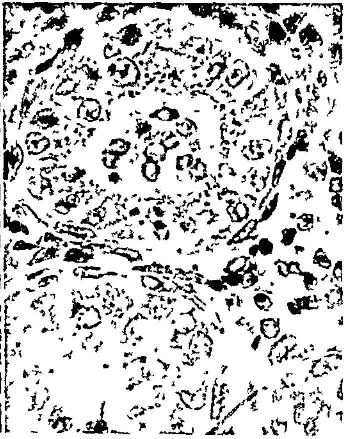

FIG 9 —Testis of tumour bearing cock Silver acetic and neutral red Ascorbic acid shown as tiny granules near lumen of tubule Wt of testis 425 mg, Ascorbic acid 31 mg /100 g wet wt ×945

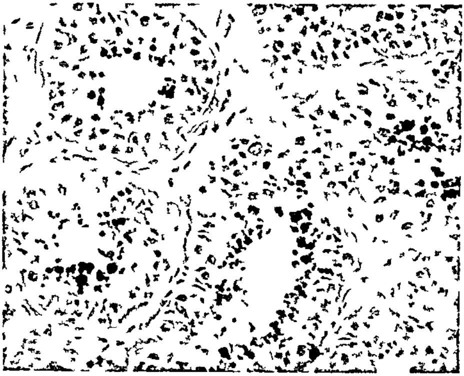

FIG 10 —Testis of tumour bearing cock stained with osmic acid and neutral red showing lipoid droplets towards lumen in degenerating tubules ×400

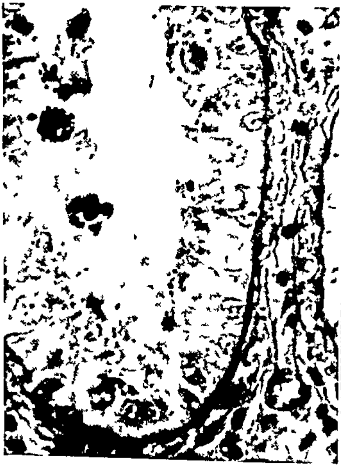

FIG 11 —Testis of tumour bearing cock stained with P A S showing fine drops of polysaccharide in the margin of the degenerating tubule and coarse drops in the desquamated cells ×945

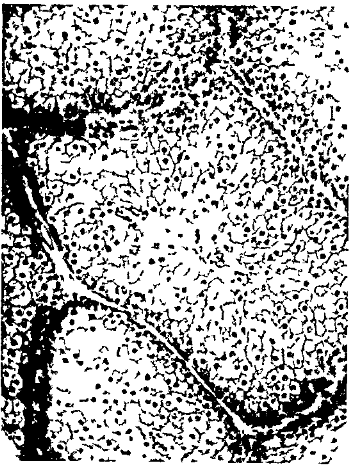

FIG 12 —Normal preen gland sebaceous zone The cells of the tubules are well preserved and the lumen is small with little secretion Wt of one lobe 740 mg Ascorbic acid 50 mg /100 g wet wt H and E ×200

in the nuclei of the cords of the testis after 24 hrs.' incubation, but the basement membranes and the cells of the stroma are strongly positive and are already stained after only 2 hrs.' incubation. Acid phosphatase stains the nuclei of the spermatic cords strongly and the chromatin network is clearly depicted after 6 hrs.' incubation. The nuclei of the spindle cells surrounding the tubules and the cytoplasm and nuclei of the interstitial cells are also stained, but less strongly. Thus the heaviest staining for alkaline phosphatase is shown by the basement membrane and by the stroma, while the highest acid-phosphatase activity is in the cells of the testicular cords.

Having regard to the well-known vagaries of these techniques and the small number of animals examined, it is not possible at present to be sure of any differences between the normal and the tumour-bearing animals.

### *Ovaries : histology and histochemistry*

The ovaries were examined with reference to the ova and the follicular cells which surround them, the spindle cells of the stroma and the strands of cells filled with lipid in the stroma which resemble the luteal cells of the mammalian ovary.

In sharp contrast with the testes, the ovaries show little change in the tumour-bearing hens. The strands of lipoid-laden " luteal " cells are usually more obvious and more numerous in the tumour-bearing birds, and the follicular cells, judging by the silver-reacting granules in their cytoplasm, contain rather more ascorbic acid. The spindle cells of the stroma show strong alkaline phosphatase activity in their nuclei and some in their cytoplasm ; the nuclei of the luteal cells are also moderately well stained, the follicular-cell nuclei only weakly after 24 hrs.' incubation. On the other hand the yolk of the ova stains weakly and both nuclei and cytoplasm of the follicular cells stain more strongly for acid phosphatase.

No marked or consistent differences are noted between the Rous-infected and the control hens. With osmic acid drops of lipid are seen in the follicular cells, and the luteal cells either have a number of black droplets or the whole cytoplasm of the cell stains with a fine black " filigree " pattern.

The P.A.S. technique clearly demonstrates the connective tissue stroma of the ovary but also stains the yolk of the ova a faint pink and gives a rather stronger diffuse red zone in the juxtanuclear cytoplasm of the follicular cells.

### *Adrenals*

The anatomical arrangement of the adrenals in fowls differs from that in mammals. The cortical and medullary cells are not separated into two clearly defined anatomical zones, the organ consisting of strands of cortical cells intermingled with strands of medullary cells.

The ascorbic-acid staining technique demonstrates these two types of tissue very clearly. The cortical cells show a few very heavy cytoplasmic silver-reacting granules, while the medullary cells have numerous but much finer granules uniformly scattered throughout the cytoplasm and tending to obscure the nucleus. The cytoplasm of the medullary cells tends to stain blue with hæmatoxylin. Frozen sections stained with Scharlach R show the cortical cells packed with lipoid droplets but sections stained with osmic acid present only a few fine black droplets in the cytoplasm.

The adrenal medullary cells stain weakly for alkaline phosphatase. Both nuclei and cytoplasm of the medullary cells stain fairly strongly for acid phosphatase, while the nuclei and cytoplasm of the cortical cells stain less strongly.

Comparison of the histological findings in the tumour-bearing and control birds reflects the haphazard nature of the biochemical results obtained for the adrenal. Sometimes the cortical cells are in broader bands in the tumour-bearing than in the control animals. This is difficult to prove by measurement because of normal variations and errors of sampling. In the birds with the larger tumours the cells of the cortical strands appear swollen and show signs of cytoplasmic degeneration. There is also a suggestion that the medullary strands are thinned, possibly by pressure. In two birds which were killed in a moribund condition (one in this series and one a bird used for passage), a surprising feature was the number of mitotic figures, suggesting damage of cortical cells, with attempted regeneration.

The osmic-acid sections were not satisfactory, as the tissues are apt to disintegrate or to stain unevenly. We could neither confirm nor deny, for Rous sarcoma in fowls, the lipoid depletion of the adrenals described by Dalton and Peters in tumour-bearing mice.

### *Preen gland*

A more detailed account of the anatomy, histochemistry and biochemistry of the preen gland (glandula uropygialis) is given elsewhere (Cater and Lawrie, 1950) but a brief note is necessary here for clarity. It is a bilobed gland, 1-1·5 cm. in length and breadth, situated just above the tail. Each lobe has a thick putty-coloured wall of radially arranged glandular tubules which discharge their secretion into the central cavity of the lobe. This secretion is a yellow oil which is discharged by a muscular duct on to the surface of a nipple-like process. Histochemical methods clearly demonstrate two zones in each lobe, (*a*) a peripheral zone, which, because it resembles closely the sebaceous glands of man, will be called the sebaceous zone, and (*b*) a central zone bordering the central cavity of the lobe. Certain cells in this zone contain glycogen and this has therefore been called the glycogen zone.

*Preen gland: sebaceous zone.* Fig. 12 shows a transverse section across a tubule in the sebaceous zone. The outer layer of cells in each tubule, lying against the connective tissue septa, are young dividing cells which stain deeply with hæmatoxylin and show fine black droplets of lipid with osmic acid. Inside this regenerating layer are situated several layers of polygonal cells, with foam-like cytoplasm due to lipoid droplets. Only the margins of these droplets stain with osmic acid, giving a fine filigree pattern. These typical sebaceous cells break down near the narrow lumen of the tubule to form the secretion, which stains intensely black for acid phosphatase after only 2 hrs.' incubation. The cells of the sebaceous zone do not stain for polysaccharide or for acid phosphatase except after 24 hrs. incubation, when the nuclei may be stained.

*Preen gland: glycogen zone.* A section across the tubule in this part of the gland shows quite a different appearance, The cells stain more deeply with hæmatoxylin and eosin and contain granules in their cytoplasm. The peripheral cells lying against the supporting connective tissue septa show brilliant red granules when stained with P.A.S. These granules have been shown by means of Best's carmine stain and by salivary amylase tests to be glycogen. These cells, as well as others lying nearer the lumen of the tubule, contain many granules which stain strongly positive for acid phosphatase after 2 hrs.' incubation, and after 24 hrs. the general blackening is such that all histological detail is obscured.

Osmic-acid staining shows the cells bordering the lumen of the tubule to be full of black droplets of lipid, in sharp contrast to the filigree pattern of the sebaceous zone.

The lumina of the tubules in the glycogen zone are wider than those of the sebaceous zone and contain secretion which stains intensely for acid phosphatase.

*Changes in the preen gland of birds with Rous sarcoma.* Early in the experiments it was noted that the preen gland of birds with Rous sarcoma is smaller, contains relatively more secretion and is usually a deeper yellow colour than that of the controls. This overall reduction in size of the preen gland in the tumour-bearing birds was subsequently shown to be statistically significant.

When the preen glands from the tumour-bearing birds were cut open they were found to contain more secretion than the controls, so that the secreting part of the gland was considerably thinned and was also yellower in colour.

Examination of sections stained with hæmatoxylin and eosin shows degeneration of the tubules of the tumour-bearing birds. The lumen of the tubules is widened, not only in the glycogen zone but also in the sebaceous zone. The secretion forms eosinophil masses, even in the most peripheral parts of the tubules lying against the capsule of the lobe. This increase of secretion in the tubular lumen is even more clearly seen in the sections stained with P.A.S., which stains the secretion red, and in sections stained

ROUS SARCOMA AND ENDOCRINE ORGANS

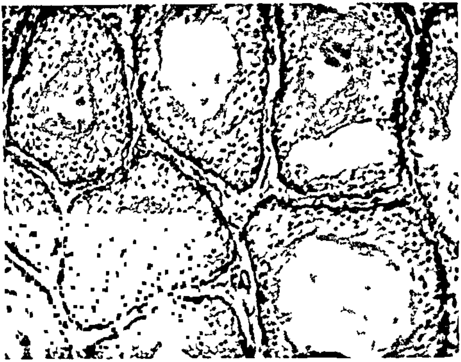

Fig. 13.—Preen gland of tumour bearing cock. The sebaceous zone shows marked degeneration of the epithelial cells and the tubular lumina are large and contain much secretion. Wt. of one lobe 290 mg. Ascorbic acid 7 mg./100 g. wet wt. H. and E. ×200.

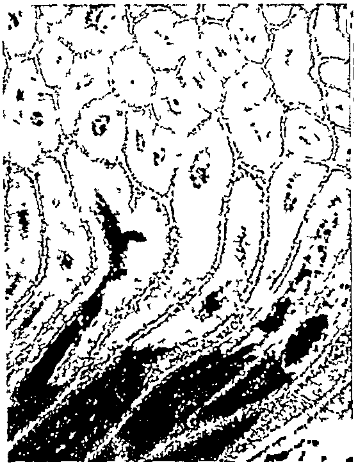

Fig. 14.—Preen gland of normal cock. Gomori technique for acid phosphatase, 24 hrs. incubation showing the sebaceous zone (above) with little secretion. Below, the glycogen zone is seen in which the cells are strongly positive. ×87.

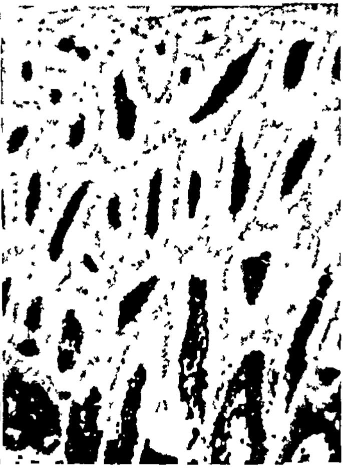

Fig. 15.—Preen gland of tumour bearing cock. Gomori technique for acid phosphatase, 24 hrs. incubation. The sebaceous zone (above) shows much secretion which has stained intense black for acid phosphatase. ×87.

the pituitaries of the tumour-bearing hens. Three tumour-bearing cocks gave 55, 57·5 and 58·5 (mean 57) per cent. acidophils and three normal control cocks, 30, 33·7 and 41 (mean 34·7) per cent. ($t$ 1·85 and $P$ 0·12) suggestive of an increase but the numbers are too few for the result to be significant.

Basophil cells were not numerous or prominent in either control or tumour-bearing birds. It is possible that the staining method employed is not the best for demonstrating basophils in the hen pituitary. However, cells with definitely blue granules were seen in which the granules were larger in size in the Rous-bearing cocks and hens. With P.A.S. these large basophil granules give a positive reaction for polysaccharide.

## DISCUSSION

Payne (1946), in a paper on the cellular picture of the anterior pituitary of normal fowls from the embryo to old age, describes an increase of the large basophils in the hen between 85 and 115 days. He also indicates that acidophil cells $A_1$ and $A_2$ are more numerous in the hen than in the cock and that they are increased by injections of œstrogen. Changes due to ageing are described at any time after 65 days, such as fusion of the granular mitochrondria of the basophils which turn into red vacuoles, acinar formation around colloid and the presence of pigment granules. This might suggest that the larger granules in the basophils of the tumour-bearing birds and the more obvious acinar formation around colloid were degenerative changes. Pearse (1949), on the other hand, suggests that the P.A.S.-positive material in the basophils and pools of colloid is gonadotropic hormone.

Guyer and Claus (1933) report that subcutaneous explants of the Flexner and Jobling carcinoma in rats cause a marked increase in the number of basophil cells in the anterior lobe of the hypophysis, similar to that which follows castration.

Apart from the changes in the pituitary there are the following definite findings :—(*a*) The testicular degeneration, with desquamation of cells into the lumen of the tubule. (*b*) In sharp contrast, the comparatively slight histological changes in the ovary, except for a suggestion of an increase in the luteal cells. (*c*) Increased secretion in the preen gland with signs of degeneration of the sebaceous zone and diminution in the size of the organ. (*d*) Some evidence that the cortical cells of the adrenal undergo cytoplasmic degeneration in birds with large tumours.

Have these findings any significance ? Are they due to the products of tumour degeneration or do they represent merely non-specific effects such as might be found in a number of different diseases ? These questions are not easily answered. To prove that these changes do not occur in any other disease would require prolonged investigation ; to find that they do occur under other conditions might still not preclude a possible significance in the pathology of the Rous sarcoma.

The first step was to search the literature for information on ovarian and testicular changes in diseases of fowls.

In Lesbouyries' monumental work "La pathologie des oiseaux" (1941), changes in the testes are described in a number of diseases, including avian tuberculosis (p. 463), *Brucella abortus* infection (pp. 404 and 405), plague (p. 263) and pseudopestis (p. 264), and the Salmonella infections (p. 369), but since in all these the ovaries are also affected, they can be ruled out of the discussion.

Lesbouyries' chapters on endocrine and deficiency diseases suggest certain causes for testicular degeneration. He quotes (p. 30) Parhon and Cahane as describing testicular atrophy following thymectomy in a duck, and draws attention (p. 84) to the importance of manganese salts in birds as necessary for the attainment of sexual maturity. Tsuji and also Bierry are quoted (pp. 47 and 48) as describing testicular atrophy and a hyperplastic reaction of the interstitial gland, but especially degeneration of the seminiferous tubules, in avitaminosis $B_1$, and Osborne and Mendel (p. 63) as describing faulty development of the testicle, with atrophy, loss of weight and a change to a deeper colour after a period of vitamin-E deficiency. In these circumstances the germinal epithelium undergoes progressive desquamation and after 4 months there is no trace of the germinal cells—nothing is left of the seminiferous tubules except the syncitium of Sertoli. This description would fit quite well the histological appearance of the testes of the cocks with Rous sarcoma. Adamstone (1931) showed that avitaminosis E affected the embryo, causing vascular degeneration and a cellular reaction of the mesoderm which brought about death—"the lethal ring".

Dam (1944) by feeding chickens on vitamin-E deficient diets was able to produce either an exudative diathesis or encephalomalacia, depending upon the quantities of protein, salts and unsaturated fatty acids in the diet. One of the striking features of the birds with large Rous tumours is the striking subcutaneous œdema, usually stained deep green, and Dam describes green colouration of the subcutaneous exudates in the E-deficient chicks. He was able to prevent the exudative diathesis by giving ascorbic acid with the vitamin-E-deficient diet, suggesting at least some relationship between the metabolism of vitamin C and vitamin E.

The suggestion that the Rous sarcoma produces a deficiency of vitamin E in the tumour-bearing birds offers a possible explanation of certain of the findings here reported. It would explain the marked testicular degeneration and the comparative lack of ovarian change. It would offer an alternative explanation of the green subcutaneous œdema, which we have hitherto regarded as the spreading margin of the tumour. On a much more tentative plane, it is not incompatible with irregularities of the vitamin-C content of the organs. That avitaminosis E should affect the preen gland, which has such intense acid phosphatase activity, is also within the realm of possibility.

That avitaminosis E might render birds more susceptible to the tumour viruses and that adequate supplies of vitamin E might have a preventive or curative effect raise interesting speculations. It has long been known that the resistance of fowls to Rous sarcoma sometimes shows marked variations for which it is not always possible to find a satisfactory explanation.

Experiments to test these possibilities are in progress and have given interesting results. Further histochemical investigation of the preen gland has also been undertaken in the hope that it might throw some light upon the synthesis of lipids or steroids, Hou having shown that the preen gland produces pro-vitamin D in birds. In addition a fuller understanding of the normal physiology of this organ is necessary before the changes produced in it by the Rous sarcoma can be understood.

## SUMMARY

The changes occurring in the testes, ovaries, adrenals, preen gland and pituitary of 3-5 months old cocks and hens bearing Rous sarcoma were investigated by biochemical analysis for ascorbic acid and by histochemical methods for ascorbic acid, lipid, polysaccharide, and acid and alkaline phosphtase.

1. Biochemical analysis shows a significant decrease in the ascorbic-acid content of the preen gland of birds with Rous sarcoma. There is a slight but significant increase in the ascorbic-acid content of the ovaries of the tumour-bearing birds. The adrenals show no significant change.

2. The Rous sarcoma gives the low value of 3-10 mg./100 g. wet weight for ascorbic acid. The matrix is free from alkaline phosphatase but stains for polysaccharide. Some of the tumour cells show cytoplasmic droplets of lipid and cells are also seen with inclusions staining for polysaccharide. The nuclear chromatin of cells in mitosis stains well for acid phosphatase ; the nuclei of other cells stain weakly.

3. The tumour-bearing cocks show marked degeneration of the spermatic cords of the testes, with thickening of the basement membrane and desquamation of the germinal cells into the lumen of the tubules.

4. The ovaries of the tumour-bearing hens show little change except for some increase of the lipid-containing cells of the stroma.

5. The cells of the spermatic cords are rich in acid phosphatase and the basement membranes and stroma have high alkaline phosphatase activity. In the ovaries the follicular cells stain moderately well for acid phosphatase, while the cells of the stroma stain strongly for alkaline phosphatase.

6. The changes in the preen gland in the birds bearing the Rous sarcoma suggest overactivity leading to degeneration.

7. The preen glands are very rich in acid phosphatase.

8. The anterior lobe of the pituitary of Rous sarcoma-bearing

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576 . 809 . 51 (*Bact. coli*) : 547 . 466 . 2 (glycine)

# OBSERVATIONS ON RESISTANCE OF *BACTERIUM COLI* TO GLYCINE

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Lysis of normal organisms by glycine was demonstrated by Maculla and Cowles (1948). Gordon, Hall and Stickland (1949) observed that organisms adapted to growth in high concentrations of glycine showed increased power of resisting lysis by glycine. The resistant and normal organisms were subcultured on to heated blood agar and then to flasks of broth. Washed suspensions of these organisms were treated with glycine solution at 37° C. and centrifuged, and the protein-nitrogen in the supernatant fluid was determined by micro-Kjeldahl.

With organisms grown in this way in peptone broth, there is some danger of reversion, and the suspension may consist in part, and to an unknown extent, of cells no longer resistant to glycine. Such cells would be expected to be lysed by glycine, and the figures in our previous paper bear this out, showing in some cases a degree of lysis of the resistant suspension. More complete resistance to lysis might be expected if suspensions were used of cells actually grown in a medium containing glycine, and experiments of this type have now been carried out.

## Material and methods

Five strains of *Bact. coli* were used, two (strains 1 and 4) which had become accustomed to grow in 6 per cent. glycine and three (strains A, B and D) which had been accustomed to grow in 4 per cent. glycine. The original strains of these organisms were grown in broth, and the resistant strains in broth containing glycine at the concentration to which they had become adapted. Both sets were incubated for 24 hours at 37° C. The resistant strains were inoculated direct into glycine broth and their growth was usually not as good as that of the normal strains.

The lysis of the bacteria was carried out as described in our previous paper. The cells were centrifuged and washed once with distilled water, and finally suspended in 10-20 times their volume of distilled water. 1·0 ml. of this suspension was measured into each of a number of tubes, and treated with 1·0 ml. of water or of an appropriate solution of the substance under test. The concentrations of the various substances given in the tables are those of the final mixture. The tubes were incubated at 37° C. for 16 hours, the bacteria removed by sharp centrifugation, and the clear or opalescent supernatant fluid treated with 0·5 ml. of 25 per cent. (w/v) trichloracetic acid solution. The protein precipitate was centrifuged out, washed once with 5 per cent. trichloracetic acid, and the amount of protein determined colorimetrically by the method of Robinson and Hogden (1940).

## RESULTS

*Glycine.* The amount of lysis is given in terms of the amount of protein found in the supernatant fluid, expressed as a percentage of the protein content of the original bacterial suspension, as determined by the method of Stickland (1951). Table I shows that the net lysis produced by glycine in the suspensions of resistant organisms is very small, as compared with that found with the normal strains, and even less than that observed with the method used in our previous paper, *i.e.* with cells grown in broth not containing glycine. A noteworthy feature is the high degree of autolysis, *i.e.* lysis in distilled water alone, found with suspensions prepared from cultures of resistant strains when grown in glycine-containing media.

TABLE I

*Lysis (per cent.)* * *by glycine of normal strains of* Bact. coli *grown in broth, and of glycine-resistant strains grown in glycine broth*

| Strain | Glycine concentration used for lysis (per cent.) | Normal strains | | | Resistant strains | | |
|---|---|---|---|---|---|---|---|
| | | Autolysis | Lysis in the presence of glycine | Net lysis due to glycine | Autolysis | Lysis in the presence of glycine | Net lysis due to glycine |
| A | 4 | 1 | 24 | 23 | 38 | 35 | 0 |
| D | 4 | 2 | 47 | 45 | 29 | 34 | 5 |
| B | 4 | 0 | 12 | 12 | 15 | 14 | 0 |
| D | 4 | 0 | 13 | 13 | 18 | 19 | 1 |
| 1 | 6 | 2 | 20 | 18 | 68 | 67 | 0 |
| 4 | 6 | 2 | 22 | 20 | 66 | 72 | 6 |
| 4 | 6 | 0 | 28 | 28 | 4 | 0 | 0 |
| D | 4 | 2 | 34 | 32 | 23 | 29 | 6 |
| | Average | 1 | ... | 24 | 32 | ... | 2 |

Suspensions of organisms were incubated overnight at 37° in water (autolysis) or in glycine solution.

* In all tables the degree of lysis is the amount of protein in the supernatant fluid expressed as a percentage of the protein content of the original bacterial suspension.

The question of the specificity of this resistance to lysis by glycine is obviously important. It was studied by observing the effect of other lytic agents on normal and resistant organisms. We studied the lysis produced by normal serum (Gordon and Hall, 1950), by sodium deoxycholate, and by an amino-acid closely related to glycine, viz. DL-alanine. Saponin and fixanol C, a synthetic detergent, were also tried but discontinued owing to technical difficulties.

*Sodium deoxycholate.* The method used to observe the degree of lysis by deoxycholate was the same as for lysis by glycine, the concentration of deoxycholate in the incubated mixture being 1 per cent. It was found that deoxycholate did not interfere with the precipitation of the protein by trichloracetic acid, nor with the determination of the protein by the biuret method. The results in table II indicate that the glycine-resistant were rather more susceptible than the normal organisms to lysis by deoxycholate.

TABLE II

*Lysis (per cent.) of normal and glycine-resistant strains by sodium deoxycholate (1 per cent.)*

| Strain | Lysis of normal strains | | | Lysis of resistant strains | | |
|---|---|---|---|---|---|---|
| | Autolysis | Lysis | Net lysis | Autolysis | Lysis | Net lysis |
| 1 | 3 | 38 | 35 | 20 | 92 | 72 |
| 4 | 1 | 30 | 29 | 7 | 93 | 86 |
| 1 | 2 | 13 | 11 | 4 | 36 | 32 |
| 4 | 2 | 11 | 9 | 5 | 37 | 32 |
| 1 | 4 | 31 | 27 | 2 | 51 | 49 |
| 1 | 2 | 22 | 20 | 12 | 59 | 47 |
| A | 1 | 25 | 24 | 4 | 48 | 44 |
| B | 3 | 24 | 21 | 11 | 28 | 17 |
| D | 2 | 31 | 29 | 4 | 40 | 36 |
| 4 | 0 | 22 | 22 | 3 | 34 | 31 |
| A | 0 | 23 | 23 | 0 | 50 | 50 |
| Average | ... | ... | 23 | ... | ... | 45 |

Suspensions of organisms were incubated overnight at 37° in a 1 per cent. solution of sodium deoxycholate. The above figures for "lysis" have been corrected for "autolysis", *i.e.* the lysis on incubation in distilled water.

The medium used for the growth of the above suspensions was peptone broth. Similar results were obtained with suspensions grown on glycine-containing broth.

A further experiment was done to observe the effect of sodium deoxycholate on normal and resistant strains, by incorporating this substance in peptone agar in varying dilutions, and observing the growth on the surface of such a medium. Bacterial suspensions were made of strains 1 and 4 in peptone water and loopfuls inoculated, the plates being incubated at 37°. After 24 hours the amount of growth was observed. The results (table III) indicate that sodium deoxycholate in this medium has a marked inhibitory effect on the growth of glycine-resistant strains, an effect which was much less marked on peptone-broth agar than on peptone agar.

*Serum.* A slight action of serum as a lytic agent on *Bact. coli* is well known, and it is of interest in this connection to mention that organisms adapted to high concentrations of glycine and subcultured in such high concentrations over many months became extremely sensitive to the lytic action of normal serum, whereas the ordinary strains of *Bact. coli* are only slightly sensitive. For example, with the resistant strains, 0·05 c.c. of a suspension containing 1500 million organisms per c.c. was almost completely inhibited by 0·1 c.c. of normal rabbit serum; whereas with the original strains 0·05 c.c. of a suspension of 100 million organisms per c.c. added to 0·1 c.c. of normal

2. Such glycine-resistant organisms, if grown in glycine media and washed, show no greater lysis by glycine than by distilled water.

3. They are also resistant to the lytic action of DL-alanine.

4. The lytic action observed with glycine and DL-alanine on normal organisms is not found with other substances tested, with the exception of Na deoxycholate.

5. Streptomycin-sensitive strains of *Bact. coli* show no lysis by streptomycin.

J. Gordon wishes to thank the Medical Research Council for a grant for expenses.

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576 . 8 . 097 . 29 : 576 . 851 . 57 (*Cl. septicum*)

# THE BETA-TOXIN (DEOXYRIBONUCLEASE) OF *CL. SEPTICUM*

G. Harriet Warrack, Ethel Bidwell and C. L. Oakley

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Eisenberg (1907) observed that cultures and filtrates of *Clostridium septicum* and *Cl. chauvoei* contained thermolabile antigens which damaged washed leucocytes incubated with them. Hanging-drop preparations of the incubated mixtures showed that the polymorphonuclear leucocytes had rounded off and lost their granules, while the nuclei had fused to form clear vesicles; in stained preparations the fragments of nuclei appeared fused into an ill-defined mass with poor staining properties. Lymphocytes and macrophages appeared less sensitive. Human leucocytes were most sensitive, then rabbit leucocytes; *guinea-pig leucocytes were relatively insensitive. Leucocidins* could be demonstrated in guinea-pig exudates due to infection with *Cl. septicum* and *Cl. chauvoei*.

Butler (1942, 1943), examining cervical smears from post-abortum cases of *Cl. welchii* infection, noted that in severe infections heavily capsulated bacilli were present and that a large proportion of the leucocytes in the smears showed severe damage. Later (1945), she commented on the fact that, in infections with anaerobic bacilli other than *Cl. welchii*, damage to leucocytes was not observed except in infections with *Cl. septicum*. Robb-Smith (1945) showed that, when *Cl. welchii* filtrates were injected into the muscles of living rabbits, karyolysis of nuclei of polymorphonuclear leucocytes and destruction of connective tissue and muscle-cell nuclei occurred in the injected area, and that similar nuclear changes could be produced in frozen sections of liver or muscle by incubating them at 37° C. with filtrates of *Cl. welchii* type A. As the material damaged in this way was Feulgen-positive—basophil Feulgen-negative material was unaffected—he concluded that the active material in the filtrates was a deoxyribonuclease. Since the capacity of organisms to produce enzymes which damaged nuclei would clearly be of considerable importance in assisting the spread of infection, it seemed worth while to examine filtrates of *Cl. welchii* and *Cl. septicum* for this capacity, and for their ability to attack deoxyribonucleic acid. Evidence has been obtained that both *Cl. welchii* and *Cl. septicum* filtrates attack sodium deoxy-precipitate was centrifuged out, washed once with 5 per cent. trichloracetic acid, and the amount of protein determined colorimetrically by the method of Robinson and Hogden (1940).

## RESULTS

*Glycine.* The amount of lysis is given in terms of the amount of protein found in the supernatant fluid, expressed as a percentage of the protein content of the original bacterial suspension, as determined by the method of Stickland (1951). Table I shows that the net lysis produced by glycine in the suspensions of resistant organisms is very small, as compared with that found with the normal strains, and even less than that observed with the method used in our previous paper, *i.e.* with cells grown in broth not containing glycine. A noteworthy feature is the high degree of autolysis, *i.e.* lysis in distilled water alone, found with suspensions prepared from cultures of resistant strains when grown in glycine-containing media.

TABLE I

*Lysis (per cent.)* * *by glycine of normal strains of* Bact. coli *grown in broth, and of glycine-resistant strains grown in glycine broth*

| Strain | Glycine concentration used for lysis (per cent.) | Normal strains | | | Resistant strains | | |
|---|---|---|---|---|---|---|---|
| | | Autolysis | Lysis in the presence of glycine | Net lysis due to glycine | Autolysis | Lysis in the presence of glycine | Net lysis due to glycine |
| A | 4 | 1 | 24 | 23 | 38 | 35 | 0 |
| D | 4 | 2 | 47 | 45 | 29 | 34 | 5 |
| B | 4 | 0 | 12 | 12 | 15 | 14 | 0 |
| D | 4 | 0 | 13 | 13 | 18 | 19 | 1 |
| 1 | 6 | 2 | 20 | 18 | 68 | 67 | 0 |
| 4 | 6 | 2 | 22 | 20 | 66 | 72 | 6 |
| 4 | 6 | 0 | 28 | 28 | 4 | 0 | 0 |
| D | 4 | 2 | 34 | 32 | 23 | 29 | 6 |
| | Average | 1 | ... | 24 | 32 | ... | 2 |

Suspensions of organisms were incubated overnight at 37° in water (autolysis) or in glycine solution.

* In all tables the degree of lysis is the amount of protein in the supernatant fluid expressed as a percentage of the protein content of the original bacterial suspension.

The question of the specificity of this resistance to lysis by glycine is obviously important. It was studied by observing the effect of other lytic agents on normal and resistant organisms. We studied the lysis produced by normal serum (Gordon and Hall, 1950), by sodium deoxycholate, and by an amino-acid closely related to glycine, viz. DL-alanine. Saponin and fixanol C, a synthetic detergent, were also tried but discontinued owing to technical difficulties.

*Sodium deoxycholate.* The method used to observe the degree of lysis by deoxycholate was the same as for lysis by glycine, the concentration of deoxycholate in the incubated mixture being 1 per cent. It was found that deoxycholate did not interfere with the precipitation

TABLE IV

*Comparison of lysis (per cent.) of normal and glycine-resistant strains of* Bact. coli *by* DL-*alanine and by glycine*

| Strain | DL-alanine concentration (per cent.) | Normal strain |  |  |  |  |  | Glycine-resistant strain |  |  |  |  |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  |  | DL-alanine |  |  | Glycine (4 per cent.) |  |  | DL alanine |  |  | Glycine (4 per cent.) |  |  |
|  |  | Autolysis | Lysis | Net lysis | Autolysis | Lysis | Net lysis | Autolysis | Lysis | Net lysis | Autolysis | Lysis | Net lysis |
| D * | 4 | 2 | 33 | 31 | 2 | 34 | 32 | 23 | 25 | 2 | 23 | 29 | 6 |
| D * | 7 | 2 | 51 | 49 |  |  |  | 23 | 25 | 2 |  |  |  |
| D * | 10 | 2 | 58 | 56 |  |  |  | 23 | 26 | 3 |  |  |  |
| A | 12·5 | 0 | 45 | 45 | ... | ... | ... | 0 | 12 | 12 | ... | ... | ... |
| B | 12·5 | 0 | 49 | 49 | ... | ... | ... | 2 | 7 | 5 | ... | ... | ... |
| D | 12·6 | 0 | 48 | 48 | ... | ... | ... | 5 | 31 | 26 | ... | ... | ... |
| D * | 12·5 | 0 | 48 | 48 | 0 | 13 | 13 | 18 | 8 | 0 | 18 | 19 | 1 |
| 4 | 12·5 | 0 | 41 | 41 | ... | ... | ... | 3 | 34 | 31 | ... | ... | ... |
| D * | 10 | 1 | 14 | 13 | 1 | 15 | 14 | 7 | 14 | 7 | 7 | 18 | 11 |
|  | Average | ... | ... | 42 | ... | ... | 20 | ... | ... | 10 | ... | ... | 6 |

* Glycine-resistant strain grown in 4 per cent. glycine broth.

Suspensions of normal and resistant organisms were incubated overnight at 37° in water or in DL-alanine solution.

rabbit serum gave no inhibition, but slight inhibition when added to 0·2 c.c. of serum (Gordon and Hall). As stated in this paper it might have an important clinical bearing in cases where organisms which have become resistant to drugs might come under the influence of the natural defences of the body.

TABLE III

*The effect of sodium deoxycholate on the growth of normal and glycine-resistant strains on peptone agar*

| Concentration of deoxycholate (per cent.) | Ordinary strains | | Resistant strains | |
|---|---|---|---|---|
| | 1 | 4 | 1 | 4 |
| Nil (control) | ++ | ++ | ++ | ++ |
| 0·1 | ++ | ++ | + | + |
| 0·25 | ++ | ++ | A number of small dry colonies, difficult to count | |
| 0·5 | ++ | ++ | | |
| 1·0 | + | + | 45 | 30 |
| 1·5 | + | + | 36 | 20 |
| 2·0 | + | + | 38 | 26 |

1 and 4 were two different strains of *Bact. coli.*
++ = heavy growth. + = good growth.
Numerals refer to number of colonies where these could be counted.

### *Effects of* DL*-alanine*

In an earlier paper (Gordon and Gordon, 1947) it was shown that glycine-resistant strains of *Shigella shigæ* can grow in concentrations of DL-alanine which would be inhibitory to normal strains. Therefore it was of interest to see how glycine-resistant strains of *Bact. coli* would behave with DL-alanine, with regard both to growth and to lysis of suspensions.

Ten normal strains of *Bact. coli* proved rather less sensitive to DL-alanine than to glycine. The lethal concentration of glycine was in the neighbourhood of 1·5-2·0 per cent., whereas with DL-alanine it was 2·5-3·0 per cent. The strains resistant to 6 per cent. glycine on the other hand grew readily in 7 per cent. DL-alanine broth.

These facts were reflected in the results when lysis by DL-alanine was measured, *i.e.* the normal strains were lysed by the concentrations of DL-alanine which prevented growth, and the glycine-resistant strains showed a resistance to lysis by DL-alanine (table IV and fig.)

In table IV it is shown that the glycine-resistant strains, when grown on a plain broth medium, are lysed by DL-alanine to an extent always less than that given by the normal strain, and when grown on a glycine-containing medium are lysed by DL-alanine only to a slight extent. The figure relates the degree of lysis of a normal strain to the concentration of DL-alanine and shows that in the growth-inhibiting range of concentrations, *i.e.* in those greater than 2·5-3·0 per cent., lysis becomes appreciable.

The concentrations of glycine and DL-alanine used in these experiments are extremely high, and it might be suspected that some part at least of their action is due to osmotic effects. For this reason a number of other substances of as varied chemical composition as possible was tested for their power to cause lysis of *Bact. coli*; the choice of substances was limited chiefly by questions of solubility. The results (table V) show that the lysis by glycine and DL-alanine is quite specific and cannot be imitated by the other substances tested.

## DISCUSSION

The above results extend our knowledge of the lysis of glycine-resistant organisms in several ways. First, it is shown that if glycine-resistant *Bact. coli* are grown on a glycine-containing medium, their subsequent behaviour with glycine shows an even greater contrast with the normal strains than when they are grown on plain broth, if allowance is made for autolysis. This autolysis, or lysis by distilled water, is sometimes considerable and we think it is to be regarded as a measure of the greater fragility of organisms growing in the glycine-containing medium. This fragility is also demonstrated by the greater susceptibility of the resistant organisms to lysis by sodium deoxycholate which can be seen even when the bacteria are grown on a glycine-free medium, and also by the extreme sensitivity to the bactericidal activity of normal serum.

Second, the question of the specificity of the lytic action of glycine has been studied. It was shown in 1947 (Gordon and Gordon) that strains of *Shigella shigæ* resistant to glycine were also resistant to DL-alanine. We have now shown that, among a number of substances tested, only DL-alanine caused lysis of normal strains of *Bact. coli* of a degree comparable with that found with glycine, and that the glycine-resistant strains are also resistant to lysis by DL-alanine. The growth-inhibition effect of DL-alanine at various concentrations on normal *Bact. coli* is paralleled by the lytic effect on suspensions. The negative results with sundry substances at high concentrations seem to point to the conclusion that this lysis is not associated with any osmotic effects.

It is of interest that with streptomycin-sensitive strains of *Bact. coli* no significant lysis by streptomycin could be observed, even at extremely high concentrations. It would appear, therefore, that the action of streptomycin on *Bact. coli* does not involve lysis.

## SUMMARY

1. Glycine-resistant *Bact. coli*, when grown on glycine-containing medium, centrifuged and washed, are more fragile than normal. This is shown by increased autolysis of the washed suspension, and by increased sensitivity to the lytic action of sodium deoxycholate and the bactericidal action of normal serum.

2. Such glycine-resistant organisms, if grown in glycine media and washed, show no greater lysis by glycine than by distilled water.
3. They are also resistant to the lytic action of DL-alanine.
4. The lytic action observed with glycine and DL-alanine on normal organisms is not found with other substances tested, with the exception of Na deoxycholate.
5. Streptomycin-sensitive strains of *Bact. coli* show no lysis by streptomycin.

J. Gordon wishes to thank the Medical Research Council for a grant for expenses.

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576 . 8 . 097 . 29 : 576 . 851 . 57 (*Cl. septicum*)

# THE BETA-TOXIN (DEOXYRIBONUCLEASE) OF *CL. SEPTICUM*

G. Harriet Warrack, Ethel Bidwell and C. L. Oakley

*From the Wellcome Research Laboratories, Biological Division, Beckenham, Kent*

Eisenberg (1907) observed that cultures and filtrates of *Clostridium septicum* and *Cl. chauvoei* contained thermolabile antigens which damaged washed leucocytes incubated with them. Hanging-drop preparations of the incubated mixtures showed that the polymorphonuclear leucocytes had rounded off and lost their granules, while the nuclei had fused to form clear vesicles ; in stained preparations the fragments of nuclei appeared fused into an ill-defined mass with poor staining properties. Lymphocytes and macrophages appeared less sensitive. Human leucocytes were most sensitive, then rabbit leucocytes ; guinea-pig leucocytes were relatively insensitive. Leucocidins could be demonstrated in guinea-pig exudates due to infection with *Cl. septicum* and *Cl. chauvoei*.

Butler (1942, 1943), examining cervical smears from post-abortum cases of *Cl. welchii* infection, noted that in severe infections heavily capsulated bacilli were present and that a large proportion of the leucocytes in the smears showed severe damage. Later (1945), she commented on the fact that, in infections with anaerobic bacilli other than *Cl. welchii*, damage to leucocytes was not observed except in infections with *Cl. septicum*. Robb-Smith (1945) showed that, when *Cl. welchii* filtrates were injected into the muscles of living rabbits, karyolysis of nuclei of polymorphonuclear leucocytes and destruction of connective tissue and muscle-cell nuclei occurred in the injected area, and that similar nuclear changes could be produced in frozen sections of liver or muscle by incubating them at 37° C. with filtrates of *Cl. welchii* type A. As the material damaged in this way was Feulgen-positive—basophil Feulgen-negative material was unaffected—he concluded that the active material in the filtrates was a deoxyribonuclease. Since the capacity of organisms to produce enzymes which damaged nuclei would clearly be of considerable importance in assisting the spread of infection, it seemed worth while to examine filtrates of *Cl. welchii* and *Cl. septicum* for this capacity, and for their ability to attack deoxyribonucleic acid. Evidence has been obtained that both *Cl. welchii* and *Cl. septicum* filtrates attack sodium deoxyribonucleate, but as considerable difficulties were encountered in dealing with *Cl. welchii* filtrates, this paper is limited to experiments with *Cl. septicum*.

### *Action of filtrates on films of fixed rabbit leucocytes*

R. G. Macfarlane (personal communication) had already drawn our attention to his observation that when methyl alcohol-fixed films of human leucocytes are incubated with filtrates of *Cl. welchii* type A for 18 hours at 37° C., the nuclei of the leucocytes fail to stain with Jenner's stain. We have found that filtrates of *Cl. septicum* also possess this property, that this activity can be inhibited by previous mixture of the filtrate with *Cl. septicum* antisera, and that such sera can be calibrated against a standard for their capacity to inhibit this indicating effect.

## METHODS

Rabbits are bled out into 1·6 per cent. sodium oxalate, the leucocytes concentrated by centrifugation and re-suspended in oxalated rabbit plasma. Trial films are made and the concentration of leucocytes adjusted to give 15-20 leucocytes per 1/12 in. objective field. Films are then made in a standard manner on microscope slides 7·5 × 1·2 cm., dried in the air and fixed in methyl alcohol. About 400 slides can be made from the blood of one rabbit; the use of large uniform batches of films greatly facilitates the comparison of results.

For the determination of the activity of filtrates against fixed nuclei, two-fold saline dilutions of filtrates in volumes of 4 ml. are placed in test-tubes; a numbered slide bearing a fixed film is placed gently in each tube so that no splashing occurs; about half the film should be submerged. The tubes, with appropriate saline controls, are incubated upright in a water-bath at 37° C. for about 18 hours; the slides are then removed, rinsed with distilled water, stained with Jenner's stain in the usual way and examined microscopically with a 1/12 in. oil-immersion objective. The upper half of the film acts as a somewhat unsatisfactory control, both for number of leucocytes per field and for efficiency of staining. As the concentration of effective material in the mixtures increases, the appearance of the leucocytes in the treated films alters. The first change is reduction in nuclear staining of lymphocytes, leading on to ghost nuclei, in which the unstained nuclei are sharply demarcated from the faintly stained cytoplasm surrounding them; polymorphs are next affected and finally eosinophils. If the concentration of effective substance is sufficiently high, no stained leucocytes may be seen in the treated film. Red cells are unaffected. Some 20 or 30 fields are examined on each slide, and the standard indicating dose determined as the amount of filtrate producing severe damage of 50 per cent. of leucocytes. A little practice soon allows the adoption of a consistent standard of reading. Most *Cl. septicum* filtrates tested had about 250 standard indicating doses per ml.

*Application to titration of sera.* A standard serum was established with an arbitrary value of 1000 units. Varying amounts of *Cl. septicum* filtrate were mixed with 1 arbitrary unit of antiserum in a total volume of 4 ml., and allowed to stand for half an hour at room temperature; they were then incubated with methyl alcohol-fixed slides of rabbit leucocytes for about 18 hours at 37° C. The test dose at one unit was taken as the amount of filtrate which, when mixed with 1 unit of antiserum, severely damaged 50 per cent. of the leucocytes in a treated film; it usually contained about 120 standard indicating doses. This test dose was then used to calibrate other sera: testing at 10 per cent. differences was easily practicable.

## RESULTS

The results are given in table I, together with the serum values against the lethal toxin of *Cl. septicum.* It is clear that the serum values in this test bear no relationship to those against the lethal toxin; it is therefore probable that the substance in *Cl. septicum* filtrates attacking fixed nuclei is distinct from the lethal toxin. We propose to call the lethal factor α-toxin, the substance affecting fixed nuclei β-toxin.

TABLE I

*Serum values against* Cl. septicum *filtrates in slide tests compared with those obtained in lethal tests, to show that the antigen effective in slide tests* (β-toxin) *is distinct from the lethal* (α) *toxin*

| Serum | Serum values (units) | | |
|---|---|---|---|
| | In slide tests against filtrate | | In lethal tests |
| | AV 1002 | AV 1003 | |
| GVP 1416 | ... | 210 | 3400 |
| GVP 1603 | 1000 | 1000 | 3200 |
| EX 630 | 100 | 110 | 1100 |
| EX 631 | 70 | 85 | 200 |
| EX 632 | 23 | 23 | 450 |
| EX 635 | 60 | 50 | 870 |
| EX 636 | 22 | 21 | 1400 |
| EX 637 | 80 | 75 | 165 |
| EX 639 | 25 | 25 | 270 |
| EX 654 | 45 | 50 | 630 |

Values obtained in 1946.

### *Enzymatic nature of the substance affecting fixed nuclei*

In view of Robb-Smith's suggestion that *Cl. welchii* type-A filtrates might contain a deoxyribonuclease and the striking effect of *Cl. septicum* filtrates on films of fixed rabbit leucocytes, we have examined a *Cl. septicum* filtrate for deoxyribonuclease and related enzymatic activities, and have compared its action on films of fixed rabbit leucocytes with those of ribonuclease and deoxyribonuclease from another source.

## METHODS

*Preparation of enzymes and substrates.* Deoxyribonuclease was prepared from ox pancreas by the method of McCarty (1945-46) and ribonuclease from a rejected fraction in the deoxyribonuclease preparation by the method of Kunitz (1940-41).

The deoxyribonuclease preparation had no action on yeast nucleic acid when tested by the method of Kunitz, but it hydrolysed fish-sperm nucleic acid (method of Laskowski, 1946) and reduced the viscosity of calf-thymus deoxyribonucleic acid. The ribonuclease preparation hydrolysed yeast nucleic acid, but had no action on fish-sperm nucleic acid.

Since the active material in the filtrate might be a depolymerase, it was essential to prepare sodium deoxyribonucleate by a method involving minimum denaturation: commercial specimens made from calf thymus were unsuitable. Sodium deoxyribonucleate was therefore prepared from calf thymus by the method described by McCarty; *i.e.* the nucleoprotein was extracted by the method of Mirsky and Pollister (1942) and deproteinisation effected by the chloroform-amyl alcohol method of Sevag *et al.* (1938).

As, under prevailing conditions, there was considerable difficulty in obtaining calf thymus in any but very small amounts, most of the work was done with deoxypentose-nucleic acid prepared from horse spleen by a modification of the method of Gulland *et al.* (1947). This preparation has been called deoxyribosenucleic acid in view of the work of Chargaff *et al.* (1949) on the deoxypentosenucleic acid of beef spleen but final proof of its identity with deoxyribosenucleic acids from other sources (*e.g.* calf thymus) is not yet available.

Sodium phenolphthalein phosphate was synthesised by the method of Huggins and Talalay (*1945*).

## RESULTS

### *Action of ribonuclease and deoxyribonuclease on films of fixed rabbit leucocytes*

Ribonuclease (1 mg./ml. acetate buffer *p*H 5) caused the nuclei to stain more deeply with Jenner's stain, presumably because it removes ribonucleic acids and allows freer access to the stain (*cf.* Stowell and Zorzoli, 1947).

The action of deoxyribonuclease was similar to that of *Cl. septicum* filtrates.

### *Enzymatic activities of* Cl. septicum *filtrates*

*Phosphatase.* Phosphatase activity was tested by incubating overnight at 37° C. a mixture of 1 ml. of culture filtrate and 1 ml. of substrate (30 mg. sodium phenolphthalein phosphate dissolved in 25 ml. acetate buffer, *p*H 5·6). Next morning 0·2 ml. *N* sodium hydroxide was added to each tube; the appearance of a very marked pink colour indicated phosphatase activity. Controls were run with uninoculated medium and with heated filtrates. Filtrates of cultures of *Cl. septicum* showed little or no phosphatase activity.

*Deoxyribonuclease. Cl. septicum* filtrates rapidly reduced the viscosity of solutions of sodium deoxyribonucleate. The relationship between concentration of substrate and viscosity was first determined as follows:—

The substrate was dissolved in 0·025 *M* veronal buffer, *p*H 7·5, containing 0·01 per cent. gelatin and 0·003 *M* magnesium as described by McCarty and the flow time determined for varying substrate concentrations. A typical curve of this relationship is given in fig. 1. (Ostwald viscometers, bulb volume 1·5 ml., capillary diameter 0·53 mm.; flow time for 2·2 ml. buffer, 50 sec.; working temperature, 37° C.).

From curves similar to fig. 1 a concentration of substrate was chosen to give a flow time of about 120 sec. Two ml. of substrate of this concentration were warmed to 37° C. in a water bath, 0·2 ml. of warmed filtrate added and the time of mixing noted. The mixture

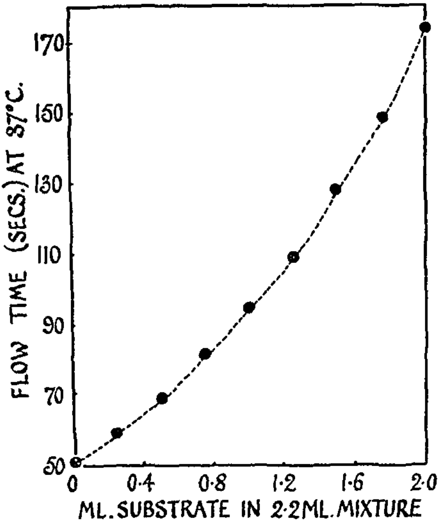

FIG. 1.—Relationship between concentration of sodium deoxyribonucleate and flow time under constant conditions.

was then transferred to the viscometer and the flow-time ($f_e$) determined 20 min. after mixing. Swyer and Emmens's (1947) flow-time function

$$v = \frac{1000(f_s - f_e)}{f_s - f_0}$$

was then calculated; $f_0$ is flow-time for buffer, $f_s$ is flow-time for substrate, $f_e$ is flow-time for substrate-filtrate mixture. Fig. 2 is a typical curve of the relationship between $v$ and filtrate concentration. Filtrates could readily be compared with one another by comparing the amounts of the filtrates producing some standard effect on the value of $v$.

The viscosity-reducing activity of *Cl. septicum* filtrates was inhibited by *Cl. septicum* antisera, but not by normal horse sera, and it was found possible to calibrate sera by making use of this inhibitory effect. Owing to the day-to-day variation in viscosity of substrate and effect of enzyme, the ordinary indicator methods in which sera are compared with a standard for their capacity to reduce the activity of a test dose of the antigen to the standard indicating effect, could not be used, and the value of the sera was recorded as the reciprocal of the amount of serum that reduced the activity of a fixed quantity of enzyme to one-half. After numerous experiments the unit enzyme was defined as that produced by 0·2 ml. of *Cl. septicum* filtrate AV 1008 (diluted 1 in 40 in 1 per cent. peptone broth plus 0·003 *M* magnesium) added to 2 ml. substrate. Values of *v* were determined for a series of multiples of this enzyme concentration, *e.g.* 1·5, 2 and 3.

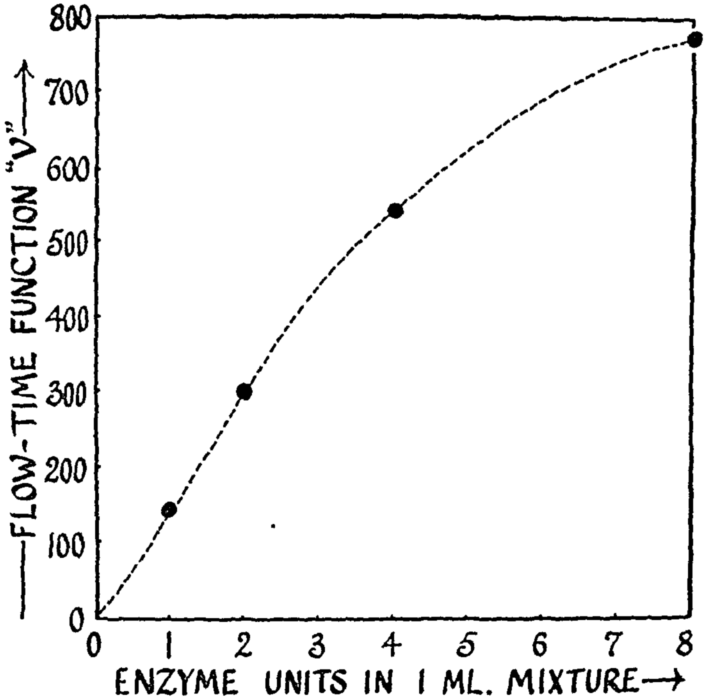

Fig. 2.—Relationship between concentration of deoxyribonuclease and Swyer and Emmens's flow-time function *v* for enzyme-substrate mixtures.

Mixtures were then made containing one volume of AV 1008 diluted 1 in 5 and 1 volume of appropriate dilutions of the antiserum; these were allowed to stand for 30 mins. 0·2 ml. of each mixture was then added to 2 ml. of substrate, warmed to 37° C. and *v* determined 20 mins. after mixing. If no antiserum was present the *v* value for the mixture would be that of 4 enzyme units; if antiserum was present *v* would correspond to less enzyme; the amount of serum present in the mixture having an activity equal to that of 2 enzyme units (flow-time $v_{50}$) could readily be determined by interpolation. Duplicate determinations on different days gave excellent agreement.

Fig. 3 gives the results for serum EX 654 and Fig. 4 those for EX 630 and GVP 1603. It will be noted that there is considerable variety in the form of the relationship between *v* and the amount of antiserum present.

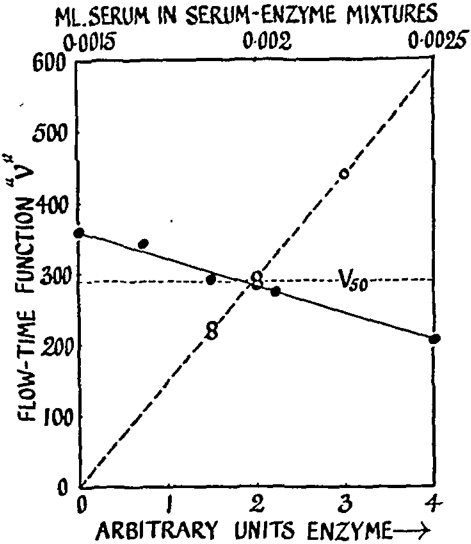

Fig. 3.—Titration of serum EX 654 for antideoxyribonuclease. Open circles, $v$ values for dilutions of enzyme ; black circles, $v$ values for enzyme-serum mixtures. $v_{50}$ is value of $v$ corresponding to half the enzyme used in the serum test.

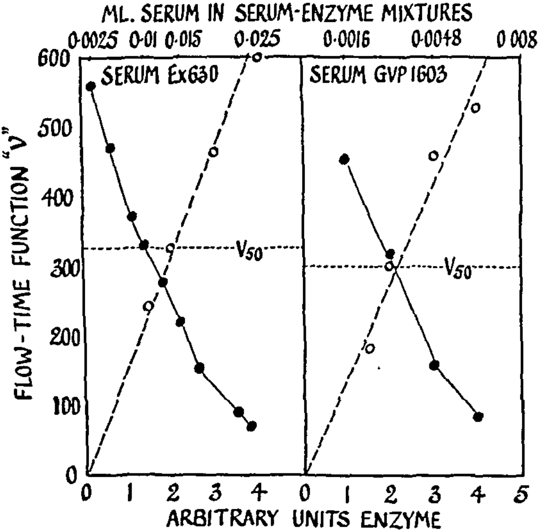

Fig. 4.—Titration of two sera for antideoxyribonuclease. Symbols as in fig. 3.

Serum GVP 1603 gave an average value of 11,000 in viscosity tests. As it had been used as standard in slide tests at a value of 1000 units all the serum values obtained in viscosity-reduction tests were divided by 11, to make the comparison with slide tests simpler. In table II the adjusted results for 8 sera are compared with those determined in slide tests. It is clear that the ratios between the values of sera obtained in the two tests are practically constant; it is probable therefore that the substance in *Cl. septicum* filtrates active in the slide test against fixed rabbit leucocytes ($\beta$-toxin) is a deoxyribonuclease. It is not a phosphatase, for the *Cl. septicum* filtrates used had little or no phosphatase activity against sodium phenolphthalein phosphate; *Cl. welchii* filtrates that contained much more phosphatase as judged by this test, had relatively little deoxyribonuclease activity.

TABLE II

*Serum values against* Cl. septicum *filtrates in slide tests compared with the values obtained in viscosity-reduction tests using sodium deoxyribonucleate as substrate, to show that $\beta$-toxin is a deoxyribonuclease*

| Serum | Serum values | | |
|---|---|---|---|
| | In slide tests against filtrate | | In viscosity-reduction tests against filtrate AV 1008 |
| | AV 1002 | AV 1008 | |
| GVP 1416 | ... | 210 | 180 |
| GVP 1603 * | 1000 | 1000 | 1000 |
| EX 630 | 100 | 110 | 100 |
| EX 637 | 80 | 75 | 90 |
| EX 639 | 25 | 25 | 31 |
| EX 640 | <8 | <5 | 6·8 |
| EX 654 | 45 | 50 | 47 |

* Standard.

### "*ACRA*" *tests for* Cl. septicum *deoxyribonuclease and its antibody*

Both the tests for *Cl. septicum* deoxyribonuclease so far described are troublesome and time-consuming. We have already shown (Oakley and Warrack, 1951) that the "ACRA" test devised by Burnet (1948) can be used to demonstrate deoxyribonuclease and to calibrate sera for specific antibody. The test is simple, and takes little time.

Serum GVP 1416, with antideoxyribonuclease value 210 units as determined by slide tests, was used as standard; the tests were done at the 0·25 unit level at 20 per cent. differences and the test dose contained about 60 standard indicating doses. The technique for calibrating the remaining sera was that given by Oakley and Warrack. The values so obtained are given in table III, together with those obtained in slide and viscosity tests; it is evident that all three methods give much the same values for the sera, and we conclude that all three methods determine the same antibody—*Cl septicum* antideoxyribonuclease, *i e* *Cl septicum* β-antitoxin

TABLE III

*Serum values against a* Cl septicum *filtrate to show that slide tests, viscosity reduction tests and modified "ACRA" tests measure the same antibody (β antitoxin), and a comparison with values obtained against* Cl welchii *filtrates in modified "ACRA" tests to show that* Cl septicum *and* Cl welchii *deoxyribonuclease are antigenically distinct*

| Serum | Serum values against | | | |
|---|---|---|---|---|
| | *Cl septicum* filtrate | | | *Cl welchii* filtrate |
| | In slide tests | In viscosity reduction tests | In ACRA tests | In ACRA tests |
| GVP 668 | 140 | | 110 | <10 |
| GVP 1363 | 160 | | 185 | |
| GVP 1416 | 210 | 180 | 210 | <10 |
| EX 630 | 70 | 90 | 60 | <1 |
| EX 637 | 75 | 90 | 105 | <1 |
| EX 639 | 25 | 31 | 27 | <1 |
| EX 640 | <5 | 6 8 | 3 | <1 |
| EX 654 | 50 | 47 | 47 | <1 |
| EX 1274 | <10 | | <2 | 420 |
| GWP 2081 | <10 | | 2 | 180 |

Values obtained in 1950

The values of the same sera against *Cl welchii* type A filtrates in "ACRA" tests are also given in table III to show that *Cl welchii* deoxyribonuclease is immunologically distinct from that of *Cl septicum*

## SUMMARY

*Cl septicum* deoxyribonuclease (β toxin) may be demonstrated in *Cl septicum* filtrates by its action on films of fixed rabbit leucocytes, by its ability to reduce the viscosity of solutions of sodium deoxyribonucleate, and by its ability, when mixed with sodium deoxy ribonucleate, to modify the "blob" formed in a modified "ACRA" test

By the use of these three indicator effects, it is possible to calibrate antisera for *Cl septicum* antideoxyribonuclease (*Cl septicum* β antitoxin) *Cl septicum* deoxyribonuclease is antigenically distinct from the deoxyribonuclease of *Cl welchii* type A

We are very grateful to Miss B Sievewright and Miss E Hart for considerable technical assistance

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# TOXIN-PRODUCING DIPHTHERIA-LIKE ORGANISMS ISOLATED FROM CASES OF SORE THROAT

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(PLATE XLVII)

A NUMBER of strains resembling *Corynebacterium diphtheriæ* in certain properties but differing in others have been isolated in this laboratory. They produce diphtheria toxin and were associated with outbreaks of sore throat suggestive of diphtheria.

In 1928 Mair described a diphtheria-like strain which differed from *C. diphtheriæ* in producing intradermal reactions in guinea-pigs which could not be prevented by diphtheria antitoxin. Diphtheria antitoxin, however, protected against the otherwise lethal effect of subcutaneous injection of the culture, although it did not prevent the formation of a local abscess. Barratt later (1933) found that the strain fermented starch and liquefied gelatin. Petrie and McClean (1934) found that Mair's strain produced a toxin immunologically identical with diphtheria toxin and a toxic element not neutralised by diphtheria antitoxin but apparently related to the toxin of *Corynebacterium ovis*. Jebb (1948) described strains in many ways similar to *C. diphtheriæ* and *C. ovis*. They could be distinguished, however, by late fermentation of trehalose, liquefaction of gelatin slopes at room temperature and failure to reduce nitrates. Cultures of these strains caused skin reactions in guinea-pigs fully protected with diphtheria antitoxin, and diphtheria antitoxin might or might not protect guinea-pigs from the lethal effect of subcutaneous injections. No certain conclusion about their epidemiological importance could be drawn.

### *Material*

The material consists of 13 strains isolated from swabs sent to this Institute for examination for *C. diphtheriæ*. The strains are designated with the following numbers:—2921/49, 2929/49, 2972/49, 728/50, 740/50, 842/50, 892/50, 903/50, 1254/50, 1329/50, 1520/50, 1605/50, and 1634/50.

## EPIDEMIOLOGICAL AND CLINICAL DATA

Strain 2921/49 was isolated at the beginning of November 1949 from a boy aged 5½ years who lived in a densely populated district near Oslo (Bærum). The patient was ill for more than a week with fever (about 38·39° C.), swelling of the tonsils and a yellowish-white membrane not extending beyond the tonsils. When the presence of diphtheria bacilli was reported, he was admitted to hospital. The submandibular lymph nodes were enlarged. He had previously been immunised against diphtheria. Strains with the same properties were repeatedly isolated from his throat.

Strain 1329/50 was isolated at the end of June 1950 from a 3½-year-old boy living in the same district at a distance of 6 kilometres from the first patient. No contact between the two was known. This patient had remittent fever up to 40° C. lasting for about a week. He had sore throat, moderately enlarged cervical lymph nodes and swollen tonsils showing discrete specks, later coalescing to a membrane, which, however, did not extent beyond the tonsils. The patient was admitted to hospital. The doctor in charge of the two patients noted that the membranes were thin and veil-like in both cases.

Strain 1605/50 was isolated from a 4-year-old boy in the same district in September 1950. He had a mild sore throat and fever, with a membrane on the tonsils. He lived on a farm about 100 metres from the home of the second patient. The disease might well have been a mild case of diphtheria, against which he had not been immunised.

Strains 2929/49 and 2972/49 were isolated in the middle of November 1949 from a 2½-year-old boy and his 7-year-old sister, living in a country district (Toten) about 200 kilometres north of Oslo. The boy had been ill for about a fortnight with sore throat and remittent fever, starting at 40·5° C. and later decreasing. A membrane covered the tonsils but did not extend beyond them. No enlargement of cervical glands was reported. The girl, who had been immunised against diphtheria several years before, had no complaint, but slight elevation of temperature (37·8° C.) was found on the evening when the swab was taken.

Strain 1520/50 was isolated in the middle of August 1950 from a man aged 30 years, living in the same district at a distance of about 20 kilometres from the last two cases. The man had been ill for two days with slight fever and sore throat. The tonsils were enlarged, but no membrane and no enlargement of the regional glands were observed. He had been immunised against diphtheria some years before.

After this report had been prepared, still another strain, apparently of the same type, was isolated from a case of sore throat, suspected of being diphtheria, in the same district. The study of this strain is not yet complete.

The 7 remaining strains were isolated from Southern Norway (Setesdal), 2 from cases of sore throat, the rest from carriers. The first strain (740/50) was isolated in March 1950 from a girl who had clinical symptoms of diphtheria, but details are not known. Strains of the same type were isolated on one or more occasions from 3 members of her family, and from one teacher and 5 pupils of schools in this locality. One of the pupils was known to have had a sore throat about one month before the girl was taken ill. The doctor in charge of the epidemiological survey states that there had been several other cases of mild sore throat in the district some time previously, but the cases had not been sufficiently severe to attract attention. He had himself treated a suspected case of diphtheria in January, and another doctor in the locality had a similar case some time afterwards. Later in the summer, there was a new case with slight symptoms—œdema of the tonsils and uvula, headache and slight fever for a few days. It seems probable that some of these cases may have been due to infection with the same organism.

In 1943 there was an epidemic of diphtheria in the district and extensive immunisation was carried out. It is known that many of the carriers, including the family of the last-mentioned patient, had been immunised at that time.

The latest case so far observed appeared in September 1950 (strain 1634/50). This was a woman in the sixties who was taken ill with fever, sore throat and an inflammatory condition of the nose. The fever—between 38 and 39° C.—lasted for about a week. The tonsils were swollen, red and succulent, but free from membrane, although material resembling diphtheria membrane was discharged from the nose. There was no definite enlargement of the regional lymph nodes. The symptoms fitted well with a diagnosis of diphtheria and it was considered that she must have been in contact with some of the carriers.

So far there has been no report of paresis in any of the patients and none of them has been seriously ill.

It should also be mentioned that typical diphtheria bacilli have not been isolated from any of the three districts within recent months.

## BACTERIOLOGICAL OBSERVATIONS

### *Morphology and cultural characteristics*

The organisms are Gram-positive pleomorphic rods, often exhibiting an almost coccal form, but diphtheria-like rods, short rods and coccoid elements may all be found in the same film. The rods sometimes resemble those of *C. diphtheriæ*, with the typical arrangement, but at other times the arrangement tends to be parallel. Metachromatic granules are present, but are often few. They are as a rule irregularly distributed in the organisms and sometimes only in one end. No spores have been observed.

Growth of the strains on different media is more abundant than that of *C. diphtheriæ*. On blood-agar after 24 hours at 37° C. the colonies have a diameter of 0·5-1 mm., and are convex, yellowish-white, and opaque, and surrounded by a narrow zone of partial hæmolysis. The colonies are friable and, when touched, tend to slide along the surface of the medium. Marked sputtering is noted if an infected loop is put into a gas flame. The colonies of *C. diphtheriæ* strains used as controls have a diameter of about half the size, are lower, greyish and more translucent, and they do not sputter in the flame.

Growth on Loeffler's serum is abundant. Ten per cent. serum-broth cultures show a surface pellicle, strong turbidity and a granular deposit. On McLeod's tellurite medium (Anderson *et al.*, 1931) well isolated, fully developed colonies (fig. 1) show a resemblance to the gravis type of colony. Thus, radial stripes may sometimes be seen and the edge of the colonies is slightly irregular although apparently to a lesser degree than with gravis. More crowded colonies are regular and smooth with a shiny or sometimes waxy lustre. The size is about the same as that of gravis colonies.

### *Biochemical properties*

In Hiss's medium the strains ferment glucose, maltose, lævulose, galactose and dextrin within 24 hours, starch within 24-48 hours and trehalose after several days—4 to 10 in different tests. Some of the strains produce weak acid in glycerol after 10 days or more.

Mannitol and saccharose are not fermented. The methyl red reaction is positive and tests for indol and acetylmethylcarbinol are negative. Gelatin stabs and slopes are slowly liquefied, starting from the second to fifth day. Nitrates are not reduced. Urea is readily decomposed. The phenylalanine reaction (Henriksen, 1950) is negative. On Tinsdale's (1947) medium the colonies are brownish-black and surrounded by a distinct brown halo, exactly like diphtheria colonies.

### *Serological and animal experiments*

The strains were tested on plates as described by Ouchterlony (1949). The trenches were filled with serum-agar containing 40 antitoxin units per ml. A Park-Williams no. 8 (PW8) strain was used as a control. All the strains produced bands of precipitate readily visible within 3 or 4 days. A turbid halo was frequently formed along the streaks of the test strains but never along the PW8 strain. The halos did not interfere with the precipitate bands and did not appear to be due to any specific precipitation but rather to a non-specific effect on the serum protein in the medium. Fig. 2 shows a plate inoculated with a representative strain from each outbreak and a PW8 strain. The precipitate bands start from the streaks of growth as straight lines and deviate towards the trench in the region where they meet the precipitate bands from the adjacent strains. The bands formed by the strains unite with each other and form an unbroken single curve. The precipitate bands of PW8 take off nearer to the trench than the bands formed by the other strains, and the crossing points with these precipitate bands are displaced nearer to the other strains, indicating that PW8 produces a more potent toxin.

Guinea-pigs and rabbits were used in the animal tests. Toxins of the strains under investigation and of a PW8 strain were obtained by filtering 9-day-old cultures in Martin's (1898, p. 32) broth prepared according to a modified formula received from the State Bacteriological Laboratory, Stockholm. Toxins of the strains 2921, 2929, 2972, 1329 and 1254 were injected in amounts of 1 ml. subcutaneously in the right thigh of guinea-pigs weighing about 250 g. Strains 2921, 2929 and 2972 were tested twice. Other guinea-pigs were inoculated subcutaneously with the same quantities of the toxins, previously incubated for 3 hours at 37° C. with 0·1 ml. diphtheria antitoxin (8000 units/ml.). All the guinea-pigs which received 1 ml. of the toxin alone died within 2 days. The post-mortem showed a hæmorrhagic area at the site of inoculation, an increased amount of fluid in the peritoneal cavity, congested and hæmorrhagic abdominal viscera and, in particular, greatly swollen and congested adrenal glands. The guinea-pigs which received antitoxin as well survived: they were killed after five days and at autopsy showed no lesions at the site of inoculation or in the viscera.

The toxins of all strains under test and 3 strains of *C. diphtheriæ* were

DIPHTHERIA-LIKE ORGANISMS

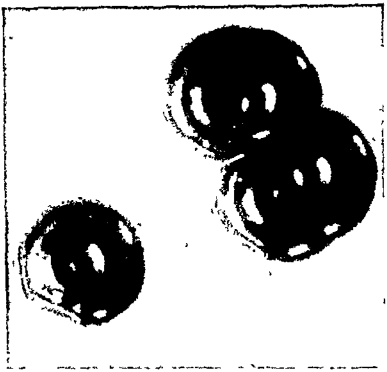

FIG. 1.—Colonies of the diphtheria-like organism on McLeod's tellurite medium. Only one of the colonies shows a slight indication of radial striping. This phenomenon often appeared to a more marked degree in well-separated colonies. ×c. 10.

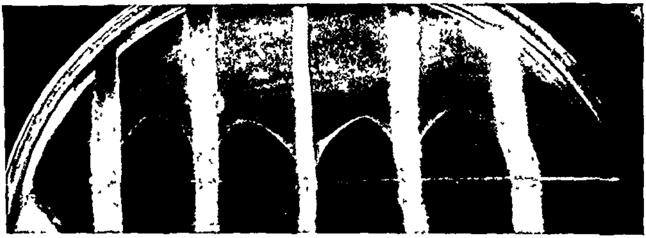

FIG. 2.—Ouchterlony plate showing precipitate bands. The central streak is the Park-Williams no. 8 (PW8) strain, the peripheral streaks are representative strains from the three different districts. The asymmetry of the precipitate bands between the PW8 and the neighbouring strains suggests that the toxin of the former is more potent ×c. 1 3.

injected intradermally into 2 sets of guinea-pigs, 4-6 injections in each, in amounts of 0·2 ml. The first set of guinea-pigs received 1000 units of diphtheria antitoxin subcutaneously on the day before the injection, whereas the second set received 30 units 3 hours after injection. On the second day, redness and slightly elevated infiltrations were evident, and necrosis had started in the skin of the guinea-pigs given 30 units of antitoxin. The necrosis increased in the next few days. From about the fifth day the lesions started to heal. The lesions at the sites injected with diphtheria toxin were more marked than those in the animals injected with the test toxins. No skin lesions occurred in the guinea-pigs which received 1000 units of antitoxin. Toxins of strains 2921, 2929, 2972 and PW8, produced in broth from the same batch, were injected in amounts of 0·1 ml. into the depilated skin of a rabbit in the dilutions 1 in 80, 1 in 160, 1 in 2000 and 1 in 8000. Observations on the second day showed that injections of the test toxins in the two highest dilutions caused a reddened area of 8-12 mm. diameter, whereas the PW8 toxin produced redness and infiltration of 12-14 mm. diameter. Correspondingly the two lowest dilutions of the test toxins produced redness and infiltration of 12-20 mm. diameter, whereas the PW8 toxin produced redness, infiltration and necrosis of 25 mm. diameter. In control tests, diphtheria antitoxin was found to prevent the skin lesions.

Intradermal tests in guinea-pigs were also performed by a similar method to that used by Jebb. Suspensions of 0·2 ml. of growth on Loeffler's slopes of all the strains under test and 3 control strains of *C. diphtheriæ*, including PW8, were used for these injections. Two sets of guinea-pigs were used, one protected with 1000 units of diphtheria antitoxin before injection and the second with 30 units 3 hours after injection. Redness, infiltration and necrosis occurred at the site of injection of the test strains in both sets of guinea-pigs. The most marked lesions occurred in those protected with 30 units of diphtheria antitoxin and injected with suspensions of *C. diphtheriæ*. Suspensions of *C. diphtheriæ* did not produce lesions in guinea-pigs protected with 1000 units of diphtheria antitoxin.

## Discussion

Diphtheria was very frequent in Norway a few years ago. Before 1939 mitis and intermedius were the most frequent types, whereas these were almost entirely displaced by gravis in the extensive epidemics during the war of 1939-45. Lately, diphtheria caused by the recognised types has decreased greatly and more extensive epidemics have not appeared. It seems reasonable to assume that the relative decrease of typical *C. diphtheriæ* may have facilitated the recognition of rare atypical strains such as those described in this paper. Still, it is striking that a great proportion of the few strains isolated during the last year from cases of suspected diphtheria were atypical organisms.

The strains here described were alike in all properties studied. Most of these properties were the same as those of *C. diphtheriæ*, from which they differed, however, in their liquefying gelatin, reducing nitrates and decomposing urea. Furthermore they showed slow fermentation of trehalose, as did the strains described by Jebb. As already stated, the strains showed a marked difference from *C. diphtheriæ* both microscopically and in the appearance of the colonies. Pleomorphism was characteristic of all the strains, although some tended to appear mostly as rods, others as coccoid elements, and variations were observed in different cultures of the same strain. The atypical strains showed a more vigorous growth than *C. diphtheriæ* on the various media.

There is ample evidence that the strains produce diphtheria toxin. Thus, on Ouchterlony plates they reacted with diphtheria antitoxin in the same manner as diphtheria strains. Further, where the toxins from a test strain and from a control strain of *C. diphtheriæ* diffused into the same area, a summation of effect was shown by the deviation and union of the precipitate bands into a single unbroken curve. The toxins could be separated from broth cultures by filtration, and, when injected subcutaneously, caused death of guinea-pigs with the same post-mortem changes as those produced by diphtheria toxin. Intradermal injections in guinea-pigs or rabbits also caused reactions similar to those of diphtheria toxin. The effects of the toxin were neutralised by diphtheria antitoxin.

Diphtheria antitoxin, however, did not give complete protection against the effect of the intradermal injection of unfiltered cultures, as it did with diphtheria strains, but only reduced the intensity of the reactions. These results suggest that some additional non-filterable, toxic factor may be present in the cultures.

The precipitate bands on Ouchterlony plates give a rough graphic estimate of the relative strength of the toxins. When it is realised that the antitoxin concentration is approximately equal at equal distances from the trench, it is obvious that differences in the position and course of individual precipitate bands are determined by the relative concentrations of toxin. Precipitate bands produced by the PW8 strain take off closer to the antitoxin trench than the bands of the test strain (fig. 2), and the crossing points with the bands of these strains are further removed from PW8 than from the other culture, indicating that the toxin of PW8 is produced in greater quantity. The strains shown in fig. 2 were selected from different districts and the graphs indicate that they produce about equally strong toxins. By comparison of the skin reactions caused by toxins produced under the same conditions, it was found that dilutions of 1 in 2000 to 1 in 8000 of PW8 toxin gave approximately the same reactions as dilutions of 1 in 80 to 1 in 160 of the toxins of strains 2921, 2929 and 2972. According to both these tests, then, the atypical strains appear to produce a weaker toxin than the PW8 strain.

The strains came from 3 widely separated districts in the country. They were isolated from both sporadic cases and healthy carriers. In one of the districts the organism was isolated from a boy who had sore throat and fever and from his sister who at the same time had a slightly elevated temperature for one evening, but no sore throat. The girl had been immunised against diphtheria some years before. In two districts sporadic cases occurred at different times and in different localities. No connection between the cases was known. In the third district there were at least two clinical cases and the organism was also isolated from 10 out of a considerable number of contacts examined. Only 6 of these strains were included in the present study. In this district extensive immunisation against diphtheria had been carried out some years before, and some of the healthy carriers, but not the patients, had been immunised. The organism was isolated on several occasions both from the patients and from some of the carriers.

The available evidence suggests that this organism can cause sore throat resembling diphtheria, although the presence of other pathogenic organisms has not been excluded. In the clinical cases associated with these strains, the lesions in the throat were less marked than is usual in diphtheria and the patients were less affected by the disease. The membranes were thin and did not extend beyond the tonsils, and there was only slight to moderate enlargement of the regional glands. The clinical course of the disease was about the same as in mild diphtheria. All the patients had fever and were confined to bed for 1-2 weeks. No severe cases were observed. This may be because of the weaker toxin production of the atypical strains as compared with the control strains of *C. diphtheriæ*. In laboratory diagnosis it would seem advisable to report such strains as diphtheria bacilli and to give the patients diphtheria antitoxin until more is known about their actual role as pathogens.

It may be difficult or impossible to detect the strains by microscopical examination of primary serum cultures, as they frequently present a coccoid appearance. The rods may be few and short, with few metachromatic granules. The growth on Tinsdale's medium is a valuable aid in the diagnosis. Further, in contrast to *C. diphtheriæ* the strains readily decompose urea. In addition, the appearance of the colonies on blood-agar and their friable consistency, the marked tendency to slide on the medium when touched and to sputter in the flame, are characteristic signs. The appearance of the colonies and the fermentation reactions might easily lead to confusion with the gravis type of *C. diphtheriæ*.

The cultural and morphological properties reported for the strains of Mair and of Jebb suggest that they may be closely related to the present strains. However, only 2 of Jebb's 26 strains gave a definitely positive methyl-red reaction. Jebb did not use the urease reaction, the phenylalanine reaction, cultivation on Tinsdale's medium or flocculation tests with diphtheria antitoxin. In contrast to the observations made with the present strains, Petrie and McClean found that a filtered broth culture of Mair's strain caused skin lesions when injected into highly protected guinea-pigs. The doses of culture injected in their experiments seemed to be approximately the same as or slightly less than those used in the tests with the present strains. Thus Petrie and McClean (p. 658) used two, one, and one-half of a subcutaneous lethal dose (0·2, 0·1 and 0·05 ml.) of a broth culture grown for 9 days before filtering. My filtrates were likewise prepared from 9-day broth cultures, and in one case the dose used for injection (0·2 ml.) was found on rough estimation to contain 2 subcutaneous lethal doses. The effect of intradermal injections of filtered broth cultures of Jebb's strains into protected guinea-pigs has not been reported. Henriksen found that the results of the urease test and the phenylalanine reaction are usually parallel in the Enterobacteriaceæ. This was not so with the atypical diphtheria strains and it may be concluded that bacteria producing urease will not always decompose phenylalanine to phenylpyruvic acid.

The atypical strains form an entity related to, but clearly distinguishable from, *C. diphtheriæ*. Their properties are also similar to those of other species in the genus Corynebacterium, but do not agree in all respects with those of any recognised species. At present it is not possible to classify the strains here described in relation to those of Jebb and of Mair. The type of organism now described certainly seems to belong to the genus Corynebacterium, but its position within the genus may be a matter of opinion.

It might be included under *C. diphtheriæ* as a separate type or variety. Practical considerations might favour this, but the proposal would necessitate some changes in the description of the species in order to admit the new type. Alternatively it might be considered as a separate species of Corynebacterium, closely related to *C. diphtheriæ*. The biochemical and morphological differences might be held to be sufficient to favour this alternative. For the present, the best position seems to be that more knowledge should be gained about these organisms and their clinical significance before a final decision is made.

## SUMMARY

A number of diphtheria-like strains have been isolated from the throat in cases of sore throat suggestive of diphtheria and from healthy contacts of these patients. The strains produce diphtheria toxin and possibly an additional non-filterable toxic factor.

They differ from typical strains of *Corynebacterium diphtheriæ* both morphologically and culturally, and in liquefying gelatin, decomposing urea, fermenting trehalose and failing to reduce nitrates. They ferment starch and show a marked resemblance to the gravis type. The strains form a uniform group, whose taxonomy is discussed.

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# NASAL CARRIER RATES OF *STAPHYLOCOCCUS PYOGENES* IN HOSPITAL NURSES

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Previous studies of the nasal carrier rates of *Staph. pyogenes* which established that these organisms can be isolated from the noses of 22-45 per cent. of healthy adults were summarised by Miles, Williams and Clayton-Cooper (1944). These workers examining both out-patients and in-patients in a Birmingham hospital found a mean carrier rate of 47·4 per cent. However, slightly higher carrier rates in the hospital population, both patients and nurses, led them to suggest that in a few nurses the hospital environment either induces the carrier state or makes manifest a latent carrier state.

Since their observations were made, the method of phage-typing of Wilson and Atkinson (1945) has allowed a more precise differentiation of the various types of *Staph. pyogenes.* In addition, recent studies (Barber and Whitehead, 1949 ; Rountree and Thomson, 1949) have demonstrated much higher nasal carrier rates for penicillin-resistant strains of *Staph. pyogenes* in the medical and nursing staff than were found in samples of the general population. Phage typing has shown that the predominant penicillin-resistant staphylococci in any particular hospital may comprise only a few phage types.

It therefore became of interest to determine at what stage in their hospital careers nurses became nasal carriers of these penicillin-resistant strains. The survey reported in the present paper was designed to secure information on this particular question. It also afforded the opportunity to study the duration of nasal carriage of particular strains, the occurrence of more than one strain in individual noses and the possible antagonistic effect of antibiotic production by certain strains of *Staph. pyogenes.*

## *The nursing population examined*

In this hospital, trainee nurses are admitted in groups every 6-8 weeks and spend six weeks in a preliminary training school before entering the hospital wards. In the hospital they are distributed in ones and twos throughout the wards. Five consecutive groups entering the preliminary training school between June 1949 and

January 1950 and comprising a total of 127 nurses were studied. On the day of their arrival at the hospital, swabs were taken from the anterior nares with swab sticks moistened with a little nutrient broth. The swabbing was repeated on the last day of the training school, after five weeks in the wards and again after an interval of a further five weeks. Each nurse was therefore examined 4 times over a period of 16 weeks.

## METHODS

Swabs were plated directly on to blood-agar plates which were then incubated for 18-24 hours at 37° C. Colonies suspected to be *Staph. pyogenes* were tested for coagulase by the slide method (Cadness-Graves *et al.*, 1943); if negative, tube tests were set up. Any obvious morphological differences such as degree of haemolysis, pigment production or roughness apparent between colonies on individual plates were noted and an example of each different colony was picked into broth. If no differences could be detected, one colony only was picked from each plate.

Further identification consisted of phage typing by the method of Wilson and Atkinson and of determination of penicillin and streptomycin sensitivity by the plate method previously described (Rountree and Thomson).

For a screening test of ability to produce antibiotic, a strain of *Staph. pyogenes* (R. 2476) isolated at the Staphylococcal Reference Laboratory, Colindale, and known to produce a large amount of antibiotic, was inoculated in a streak across the centre of a 10 per cent. horse-serum agar plate. After 24 hours' incubation, the plate was then inoculated with loopfuls of broth cultures of the strains to be tested by streaking at right angles to the primary inoculum. A control culture of the penicillin-sensitive Oxford H strain of Staphylococcus was included on each plate. After a further 24 hours' incubation the zones of inhibition of growth produced by R. 2476 were measured and compared with that of the H strain. Preliminary tests had shown that strains in which no inhibition of growth occurred were themselves producers of antibiotic. It was therefore assumed that absence of inhibition by R. 2476 indicated antibiotic production by the non-inhibited strain. All strains were dried soon after isolation and stored in case it should be required to compare them with further strains isolated from the same nurse.

Since the validity of conclusions drawn from results obtained by examining only one colony from each positive plate depended on the assumption that only one specific type of *Staph. pyogenes* was present in the nose at a particular time, it was desirable to find out what proportion of plates might contain more than one type. This information was obtained partly by the observations of differences in colony appearance. Of 280 positive plates examined, 7 (2·5 per cent.) obviously contained two types of colony which were subsequently differentiated by phage typing. Two of these plates came from the same individual. However, a further check was made of a proportion of the plates on which the colonies showed no morphological differences. After the phage type of the original colony picked had been ascertained, further colonies were tested for phage type using only the phages to which the first colony had been susceptible. If no reactions were obtained, the cultures were re-typed with the complete range of phages. A total of 80 plates was examined in this way. From 69 of them at least 10 colonies were picked. From the remaining 11 plates, which did not contain as many as 10 colonies, all colonies present were examined. In 6 of the 80 plates (7·5 per cent.) two distinct phage types were detected; two of these plates came from the same individual. In the remainder of the 80 plates, only one phage type was detected on each plate.

Combining these two sets of observations we found that in 13 (4·5 per cent.) of 280 positive plates, two phage types of *Staph. pyogenes* were detected. The exact proportion of nurses examined who carried more than one type was, however, more difficult to estimate accurately. To do this, many colonies from every plate would have had to be examined. This was impracticable for various reasons: 35 of the strains examined were not typable; some plates grew only 1 or 2 colonies, and the volume of work required would have been too great for the facilities available. Taking into account the figure of 7·5 per cent. derived from the 80 plates examined, we arrived at an estimate of 10 per cent. which probably errs on the high side. Since 4·5 per cent. of these double carriers were actually detected by the methods used, the maximum error present in our results with regard to double carriers should not be more than 5 per cent.

## RESULTS

### *Crude carrier rate*

Table I shows the carrier rates observed in the five groups of nurses examined, together with the totals. The 127 nurses entering the training school had a carrier rate of 53·5 per cent. with 5 (4 per cent.) carrying penicillin-resistant strains and 1 a strain resistant to both

TABLE I

*Nasal carrier rates of* Staph. pyogenes, *of penicillin-resistant strains of* Staph. pyogenes *and of penicillin- and streptomycin resistant strains of* Staph. pyogenes *in five groups of trainee nurses entering hospital between June 1949 and January 1950*

| | Group | | | | | Total | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | No | Per cent |
| (1) *Entry into training school* | | | | | | | |
| Number | 36 | 19 | 23 | 13 | 36 | 127 | |
| Carriers of *Staph. pyogenes* | 19 | 8 | 14 | 10 | 17 | 68 | 53·5 |
| Penicillin resistant | 1 | 0 | 1 | 2 | 1 | 5 | 4 |
| Streptomycin- and penicillin-resistant | 0 | 0 | 0 | 1 | 0 | 1 | 0·8 |
| (2) *End of training school* | | | | | | | |
| Number | 33 | 19 | 22 | 11 | 31 | 116 | |
| Carriers of *Staph. pyogenes* | 14 | 7 | 16 | 9 | 15 | 61 | 52·6 |
| Penicillin resistant | 0 | 1 | 2 | 1 | 1 | 5 | 4·3 |
| Streptomycin- and penicillin-resistant | 0 | 0 | 0 | 0 | 0 | 0 | .. |
| (3) 5 *weeks in wards* | | | | | | | |
| Number | 32 | 19 | 21 | 10 | 30 | 112 | |
| Carriers of *Staph. pyogenes* | 23 | 14 | 18 | 8 | 17 | 80 | 71·4 |
| *Penicillin resistant* | 14 | 8 | 4 | 3 | 7 | 36 | 32·1 |
| Streptomycin- and penicillin-resistant | 3 | 6 | 2 | 3 | 4 | 18 | 16·0 |
| (4) 10 *weeks in wards* | | | | | | | |
| Number | 30 | 18 | 21 | 8 | 27 | 104 | |
| Carriers of *Staph. pyogenes* | 22 | 12 | 14 | 8 | 15 | 71 | 68·1 |
| *Penicillin-resistant* | 15 | 7 | 4 | 4 | 10 | 40 | 38·5 |
| Streptomycin- and penicillin-resistant | 1 | 7 | 2 | 1 | 4 | 15 | 14·4 |

penicillin and streptomycin. At the end of the training school 116 nurses remained and little change in the crude carrier rates was apparent, 52·6 per cent. carrying *Staph. pyogenes*, 4·3 per cent. penicillin-resistant strains and none streptomycin-resistant strains. After five weeks in the wards, 112 nurses were available for examination. Eighty (71·4 per cent.) of these were now carriers. From 36 (32·1 per cent.) penicillin-resistant strains were isolated and 18 (16 per cent.) carried strains resistant to both penicillin and streptomycin. After 10 weeks in the wards, 104 nurses were available for examination, 71 (68·1 per cent.) of whom were carriers. From 40 (38·5 per cent.) penicillin-resistant strains were isolated and from 15 (14 per cent.) strains resistant to both penicillin and streptomycin.

It was apparent therefore, that a period of five weeks in the wards had raised the carrier rate from 52·6 to 71·4 per cent., the penicillin-resistant carrier rate from 4·3 to 32·1 per cent. and the streptomycin-resistant carrier rate from 0 to 16 per cent. No significant changes in crude carrier rates had occurred in the training school or during the second period of 5 weeks in the wards. The same trends occurred in each of the five consecutive groups.

### *Detailed analysis of the carrier rate*

Of the 127 nurses admitted to the training school 104 were still in the hospital at the final swabbing. The results of these 104 nurses are therefore suitable for detailed analysis. The figure shows the overall carrier rates of these 104 nurses, which did not differ substantially from the crude carrier rates given in table I. The results have been analysed with reference to the frequency of positive swabs, duration of carriage of particular strains, the relationship of environment to changes in the carrier state, association of change in type carried with antibiotic production, and the distribution of phage types among the strains isolated.

It should be noted that a number of strains were not typable with the phages available. However, if successive non-typable strains from the same person showed identical colonial appearances, sensitivity to penicillin and streptomycin and degree of inhibition by R.2476, they were regarded as being identical. In cases of doubt, the previously isolated dried strains were opened and compared with these freshly isolated.

### *Frequency of positive swabs*

Table II gives the analysis of the number of occasions on which positive swabs were obtained from the same individual among 104 nurses. It also shows in how many of those giving positive swabs a change in strain occurred at subsequent swabbings.

Sixteen (15·4 per cent.) were consistently negative. Thirteen (12·6 per cent.) were positive on one occasion only. Of these, 7

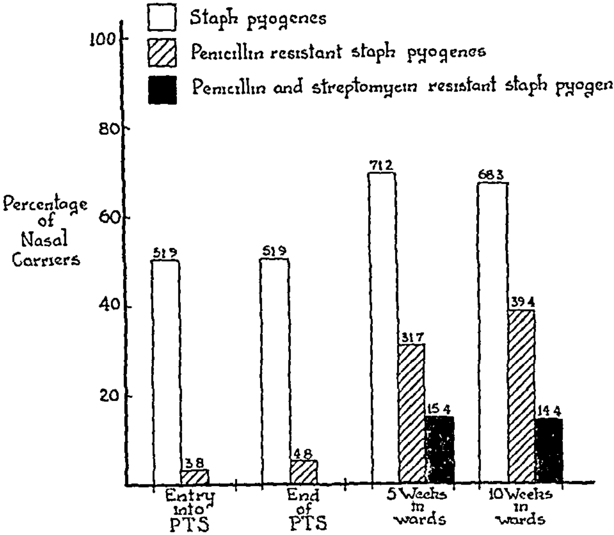

FIG.—Nasal carrier rates of *Staph. pyogenes*, of penicillin resistant strains of *Staph pyogenes* and of penicillin- and streptomycin resistant strains of *Staph pyogenes* in 104 trainee nurses swabbed on 4 occasions in 16 weeks P.T S. = preliminary training school.

TABLE II

*An analysis of the nasal carrier state of* 104 *nurses each swabbed on* 4 *occasions over a period of* 16 *weeks*

| Observation | Number | | Per cent * |
|---|---|---|---|
| Negative on 4 occasions | 16 | | 15 |
| Positive on 1 occasion | 13 | | 13 |
| Positive on 2 occasions | 21 | | |
| (*a*) same strain | | 11 | 20 |
| (*b*) different strain | | 10 | |
| Positive on 3 occasions | 16 | | |
| (*a*) same strain | | 6 | 15 |
| (*b*) 2 different strains | | 8 | |
| (*c*) 3 different strains | | 2 | |
| Positive on 4 occasions | 38 | | |
| (*a*) same strain | | 29 | 37 |
| (*b*) different strain | | 9 | |

* To nearest whole number Corresponding figures in text given to first place of decimals

showed only a few colonies, four gave a large number of colonies followed by negative swabs, and in two large numbers of colonies were obtained at the final swabbing. The majority of these single positive swabs can be regarded therefore as the result either of contamination of the nose just before swabbing or of only temporary colonisation. Twenty-one (20·2 per cent.) yielded two positive swabs. In 13 of these, the positive swabs were obtained on consecutive occasions after the nurses had gone into the wards. From 9 the same strain was isolated on both occasions and these positive cultures can be regarded as the result of colonisation of the nose consequent upon exposure to the staphylococci of the ward environment. All save one were penicillin resistant. From the remaining 4 of these 13 nurses different strains were isolated at each swabbing but all were penicillin resistant.

In 2 cases two positive swabs, both of the same type, were *obtained in the training school and were followed by two negative* swabs. In the remaining 6 nurses a first or second positive swab was followed by a negative, and at the 3rd or 4th swabbing a different strain was obtained. There were no cases in which the same strain could be identified from two positive swabs separated by a negative swab. This suggests that in intermittent carriers a strain previously present not detected at subsequent swabbing is unlikely to appear again unless re-introduced from the environment. From 16 (15·4 per cent.) nurses three positive swabs were obtained. In 6 of these the same strain was detected on 3 consecutive occasions. In 8 nurses different strains appeared after a negative result and in 2 three different strains were obtained on 3 different occasions. These last 10 nurses may therefore be regarded as intermittent carriers.

Four positive swabs were obtained from 38 (36·5 per cent.) of the nurses. From 29 the same strain was isolated on each occasion, that is, each nurse was a constant carrier of the same type during the 16 weeks she was observed. A change in the type of strain carried occurred in 9 nurses. From 8 of them a penicillin-resistant strain was isolated at the third or fourth swabbing. In the ninth nurse different strains were isolated at the first two swabbings while at the third and fourth swabbings two further strains were present together on each occasion.

## ENVIRONMENT AND CHANGES IN THE CARRIER STATE

*Changes in the preliminary training school.* The six weeks spent in the preliminary training school served as a control period during which we could assess changes in the carrier state of individuals not exposed to the specific influence of the ward environment, although closely associated in the classrooms of the school. Although the crude carrier rates at the beginning and end of the school were identical, examination of the individual histories showed that some changes had in fact taken place. Of the 50 non-carriers on entry, 8 had become carriers; and of the 54 carriers, 8 had become non-carriers and one had changed in type. Nine of 104 nurses, therefore, had acquired new strains in the training school. These 9 nurses were scattered throughout the five groups and a different strain was isolated from each. Seven of the strains were penicillin sensitive. Two were penicillin resistant and it is perhaps of some significance that one of these was obtained the day after the nurse concerned had returned from spending a week in the nurses' sick bay in the main part of the hospital. There was no evidence, therefore, that the environment of the training school tended to give rise to the carrier state or that any particular strain was widespread there. There also appeared to be little evidence of exchange of nasal strains between individuals living and working in fairly close contact in the school.

*Changes in the hospital wards.* At the end of 5 weeks in the wards, the position was as follows. Of the 54 carriers entering the wards, 8 had become non-carriers, 37 were still carrying the same strain as before and 9 had changed in type. Twenty-eight of the 50 non-carriers had become carriers, and negative swabs were obtained from 22. Thirty-seven (35·5 per cent.) of the 104 nurses had therefore acquired new strains in the wards, compared with 9 (8·6 per cent.) in the training school. This difference of 26 per cent. between wards and training school is highly significant ($\chi^2 = 21{\cdot}88$, $P < 0{\cdot}001$). Twenty-seven of the 37 strains acquired in the wards were penicillin resistant.

At the end of the second five weeks in the wards further changes had taken place. Of the 74 carriers, 50 were still carrying the same strain, 13 had become non-carriers and in 11 there had been a change in type. Of the 30 non-carriers at the beginning of this period, 10 had become carriers. Nineteen of the 21 strains acquired during this period were penicillin resistant.

During the 16 weeks of the investigation there was a proportion of carriers, therefore, who became non-carriers. This tendency was not correlated with environment since the proportion of carriers becoming non-carriers during the period in the training school and during the two periods of five weeks in the wards were 14·9, 14·9 and 17·4 per cent. respectively.

One further question can be answered by a study of the individual histories. When exposed to staphylococci of the ward environment did non-carriers acquire such strains more readily than those who were already carriers on entering the wards? During the first 5 weeks in the wards 28 (56 per cent.) of the 50 non-carriers became carriers, compared with 9 (16·6 per cent.) of 54 carriers who acquired strains different from those previously carried. This difference of 39·4 per cent. in favour of non-carriers acquiring ward strains is highly significant ($\chi^2 = 17{\cdot}48$, $P < 0{\cdot}001$). When the second period in the wards is considered the difference is not so great, 11 (14·9 per cent.) of 74 carriers acquiring different strains compared with 10 (33 per cent.) of 30 non-carriers becoming carriers. This difference of 18·1 per cent. is significant ($\chi^2 = 4{\cdot}45$, $P < 0{\cdot}05$) but it suggests that by this time the non-carriers consisted chiefly of those who possessed a high degree of resistance to colonisation of their noses by *Staph. pyogenes*.

## PRODUCTION OF ANTIBIOTIC BY THE NASAL STRAINS

Some strains of staphylococci are known to produce an antibiotic capable of inhibiting the growth in artificial media of Corynebacteria and of other strains of staphylococci. Jennings and Sharp (1947) found that 23 of 202 strains of staphylococci examined were actively inhibitory for *Corynebacterium xerosis* and that most of these 23 strains also inhibited *Staph. aureus*, strain H. The possibility of a correlation between ability of a strain of *Staph. pyogenes* to produce antibiotic and its ability to replace in the nose another strain not producing antibiotic is therefore of considerable interest.

Two sets of data might be expected to provide evidence of such a correlation, namely, those in which there had been a change in the strain obtained at consecutive swabbings and those in which two distinct strains had been isolated at a single swabbing and in which the subsequent history was available.

There were 21 occasions on which different strains were isolated at successive swabbings. In one nurse both strains were antibiotic producers. In 13 nurses both strains were antibiotic-negative. In 2 nurses an antibiotic-negative followed an antibiotic-positive strain and in the remaining 5 an antibiotic-positive followed an antibiotic-negative strain. It is impossible, however, to be certain that in the interval between swabbings the individual did not become a non-carrier and then subsequently acquire the new strain. The consistency of the values obtained for conversion to the non-carrier state contrasted with the increase in carrier rate during the periods in the wards suggests that such an occurrence was probably not frequent; but the evidence offered by this set of data is inconclusive.

There were 13 occasions on which two strains were isolated from the same swab. On 5 occasions both strains were antibiotic-negative. On 8 occasions one strain was an antibiotic producer capable of inhibiting on artificial media the growth of the other strain. In two of the nurses the pair of strains was isolated at the final swabbing and no further information was available. In one case neither strain was isolated at the next swabbing. In 3 cases the surviving strain was antibiotic negative and in 2 cases it was an antibiotic producer.

The results, admittedly obtained with a very small number of strains, do not provide any basis for the hypothesis that the ability to produce an antibiotic substance in artificial media endows a strain with the ability to supplant a non-antibiotic-producing strain already present on the nasal mucosa.

### *Phage types of* Staph. pyogenes *isolated*

The association of phage type with antibiotic production and with penicillin- and streptomycin-resistance in the 149 distinct strains isolated is shown in table III. Where the same strain was isolated on successive occasions from an individual it was counted only once in compiling the table.

TABLE III

*Distribution of phage types, antibiotic production and resistance to penicillin and streptomycin in* 149 *strains of* Staph. pyogenes *isolated from the noses of* 127 *nurses*

| Phage type | Number | Antibiotic producers | Penicillin resistant | Streptomycin resistant |
|---|---|---|---|---|
| 3A/3B/3C/51 . . | 23 | 19 | 0 | 0 |
| 6, 6/47 . . . | 8 | 0 | 1 | 0 |
| 7/47 . . . . | 15 | 9 | 12 | 3 |
| 29/44/52 . . . | 14 | 0 | 0 | 0 |
| 31B . . . . | 18 | 3 | 18 | 9 |
| 42D . . . . | 2 | 0 | 1 | 0 |
| 42F . . . . | 5 | 1 | 3 | 2 |
| 47, 47/31B . . | 20 | 0 | 17 | 10 |
| 47A . . . . | 1 | 0 | 0 | 0 |
| 47C . . . . | 8 | 4 | 3 | 0 |
| Not typable . . | 35 | 5 | 5 | 2 |
| Totals . . | 149 | 41<br>(27·5 per cent.) | 60<br>(40·3 per cent.) | 26<br>(17·4 per cent.) |

Forty-one (27·5 per cent.) of the strains were antibiotic producers, but it is noteworthy that strains lysed by one or more of the closely related phages 3A, 3B, 3C and 51 provided a large proportion of these, 19 of 23 strains belonging to this group being antibiotic producers. Nine of 15 strains belonging to type 7/47, 4 of 8 belonging to type 47C and 3 of 18 belonging to type 31B produced antibiotic. All these types are closely related. Five of the non-typable strains were antibiotic producers, 4 of these being coagulase-positive *citreus* strains. No strains belonging to the group lysed by the related phages 29, 31, 44 or 52 or to types 6, 6/47, 47 or 47/31B produced antibiotic. There is a fairly close association, therefore, between phage type and the ability to produce antibiotic.

It has already been noted (Barber and Whitehead, 1949 ; Rountree, Barbour and Thomson, 1951) that penicillin-resistant and streptomycin-resistant strains of *Staph. pyogenes* tend to belong to the group lysed by phages 6, 7 and 47 or by related phages. This association was also observed during the present investigation and the predominance of resistant strains of types 7/47, 31B, 47 and 47/31B is shown in table III. At the same time, however, this predominance is a reflection of the fact that these strains of staphylococci were also those most frequently isolated from the hospital environment, being found in many patients cross-infected in hospital and in the air and dust of the wards.

## DISCUSSION

The investigations of Miles *et al.* (1944) and of Williams (1946) indicated that people might be persistent nasal carriers, persistent non-carriers or intermittent carriers of *Staph. aureus*. Our observations have confirmed their findings and the phage-typing method has provided some additional information on persistent and intermittent carriers. The persistent carriers tended to carry always the same strain, 29 of 38 carrying the same strain for at least 16 weeks. With the intermittent carriers, it was found that strains isolated after an interval of non-carriage were of different type from those previously present, indicating that a negative swab is not the result of failure to detect organisms actually present but is indeed due to the absence of *Staph. pyogenes* from the nasal mucosa. Increases in carrier rates consequent upon hospitalisation can therefore be regarded as the result of implantation in the nose of hospital strains and not of activation of a latent carrier state.

Only a small number of nurses carried more than one type of *Staph. pyogenes* on any particular occasion. Furthermore, although the nurses were exposed to a ward environment heavily contaminated with staphylococci, it was noteworthy that a large proportion of those who were already carriers on entering this environment did not acquire the ward strains. Implantation of the penicillin-resistant ward strains occurred chiefly in those who had been non-carriers or intermittent carriers. These facts suggest that the presence on the nasal mucosa of one strain of *Staph. pyogenes* is a reasonably effective, although not absolute, barrier to the establishment of an added strain. The mechanism of this "exclusion" is unknown and no evidence could be found that it was correlated with the production of antibiotic by the already established strain since only 5 of the strains isolated from the 29 persistent carriers of the same strain were antibiotic producers.

The actual source of the strains implanted in the nurses' noses while they were in the wards is of some interest. Since there was little evidence of any exchange of nasal flora in the training school, direct infection from other more senior nurses in the wards already carrying the hospital strains was unlikely. On the other hand, infected dressings from patients, bedclothes and dust are known to be potent sources of these staphylococci. The junior nurses do not handle patients' dressings but their duties include the making of beds. Air samples taken in the wards during bedmaking have shown large counts of *Staph. pyogenes* of the types found in the nurses' nasal swabs. Implication of the patients' bedclothes as the source of the nurses' strains is therefore obvious.

Williams (1946) has pointed out that the statement that the average nasal carrier rate for *Staph. aureus* is in the region of 50 per cent. does not give a complete picture of the magnitude of the nasal reservoirs of staphylococci. He found that 80-90 per cent. of people examined over a period of 10 weeks were nasal carriers on one or more occasions. Our figures for a period of 16 weeks showed that only 15·4 per cent. of the nurses were persistently negative, and agree well with those of Williams. Whether swabbings at more frequent intervals would have produced more positive results cannot be determined. However, the numbers of the negative carriers and of those positive on only one occasion suggest that only a small proportion of people possess absolute resistance to colonisation of the nasal mucosa by *Staph. pyogenes.* That this resistance increases with age is indicated by the fact that 90 per cent. of babies born in hospital become heavy nasal carriers within 7 days (Cunliffe, 1949; Rountree and Barbour, 1950). Moreover, observations still in progress on carrier rates in older hospital patients exposed to the same ward environment as the nurses have shown that few of these people became carriers of the hospital strains.

## SUMMARY

A study has been made of the nasal carrier rate of *Staph. pyogenes* in first-year trainee nurses, primarily to determine at what stage in their hospital career they became carriers of penicillin-resistant strains.

Within 5 weeks of their entering the hospital wards, the nasal carrier rate rose from 52·6 to 71·4 per cent. and the rate for penicillin-resistant strains from 4·3 to 32·1 per cent. Compared with those who were already carriers of *Staph. pyogenes* on entering the wards, a greater number of non-carriers acquired the penicillin-resistant ward strains.

Analysis of the carrier rate obtained at 4 separate swabbings over a period of 16 weeks showed that 15·4 per cent. of 104 nurses were persistent non-carriers while 36·5 per cent. were persistent carriers. The majority of the persistent carriers always carried the same phage type of *Staph. pyogenes.* At each swabbing a certain proportion (15-17 per cent.) of previous carriers had become non-carriers.

At least 90 per cent. of the carriers carried only one type of *Staph. pyogenes* on any given occasion. The presence on the nasal mucosa of an established strain appeared to impede the establishment there of any other strain. There was no evidence that this phenomenon could be associated with the ability of a given strain to produce antibiotic in artificial media. Ability to produce antibiotic was found, however, to be associated with certain phage types.

This investigation was supported in part by a grant from the Australian National Health and Medical Research Council.

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# SHORT ARTICLES

578 . 636

## A SIMPLE TECHNIQUE FOR MAKING SMEARS OF DEPOSITS FROM SMALL QUANTITIES OF FLUID EXUDATES

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THE purpose of this publication is to describe a very simple method of preparing satisfactory smears from exudates such as cerebrospinal fluids in tuberculous meningitis The method we have used in the past and which is generally adopted is to spin the cerebrospinal fluid for one hour at 3000 r p m and make smears from as much of the deposit as can be obtained by scraping the bottom of the centrifuge tube with a bacteriological loop For some time, however, it has been felt that in spite of attempts to secure the whole deposit from these fluids, some of it must be left behind and possibly some " positive " tuberculous meningitis fluids may have been missed Accordingly, the following technique has been evolved

A hard glass tube into which a ¾ inch circular coverslip will just fit, is chosen Non nutrient agar at a strength of 6 per cent is added in sufficient quantity to fill the rounded bottom of the tube On cooling, a tough flat surface is present at the bottom of the tube A coverslip marked to indicate the correct side is dropped into the tube and the cerebrospinal fluid added The whole is then centrifugalised in the usual way and, when the tube is removed from the centrifuge, the supernatant fluid can be poured off, leaving the coverslip, with the deposit firmly adherent to it, slightly adhering to the agar The cover slip is then gently removed quite easily by means of a bent probe and attached to a microscope slide prepared by placing a small quantity of canada balsam on top, then heating the slide until the canada balsam is fairly fluid The coverslip is placed on the canada balsam and by the time the slide has cooled it is firmly adherent Before attaching the coverslip it is gently warmed to dry it and the preparation fixed by heat after placing the coverslip on the end of the microscope slide The preparation is stained in the usual manner and our experience so far is that the coverslip does not detach during staining It has been found that the deposit is a thin even smear which is easily examined with the advantage that the edge of the preparation is clearly defined by the edge of the coverslip It seems justifiable to assume that almost the whole deposit from the cerebrospinal fluid is recovered by this method

After use, the tubes are cleaned by removing the agar, boiling in water, brushing with soap and water, rinsing and autoclaving

### *Summary*

A simple method of obtaining the whole deposit from small quantities of fluid exudate, particularly cerebrospinal fluid from cases of tuberculous meningitis, is described

578.65

# A METHYL GREEN-PYRONIN-ORANGE G STAIN FOR FORMALIN-FIXED TISSUES

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Since the introduction of methyl green-pyronin staining mixtures by Pappenheim (1899) most workers have preferred alcohol to formalin as a fixative for material to be stained in this way (Schmorl, 1934; Conn and Darrow, 1947). Formalin is a far more satisfactory anatomical fixative than alcohol and this paper describes a methyl green-pyronin-orange G stain which gives beautiful and consistent staining of *formalin-fixed* tissue sections. The effect is that given by Pappenheim staining of alcohol-fixed material, without the distortion resulting from alcohol fixation, and with the additional staining of acidophil elements with orange G. The method is a simple progressive one, using a single staining solution.

## *Solutions*

N.B. "Water" and "aqueous" refer to distilled water unless otherwise stated.

*Fixative*

| | | |
|---|---|---|
| Formalin (A.R.) | | 1250 ml. |
| Saturated solution NaCl in tap water | | 270 ml. |
| Precipitated chalk ($CaCO_3$) | about | 10 g. |
| Tap water to | | 10 l. |

Shake well before pouring out.

*Stain*

Solution A

| | |
|---|---|
| 5 per cent. aqueous pyronin (see Note 1) | 17·5 ml. |
| 2 per cent. aqueous methyl green (see Note 2) | 10·0 ml. |
| Water | 250 ml. |

Solution B

| | |
|---|---|
| 1·0 per cent. aqueous orange G | 30 ml. |
| M/5 acetate buffer, *p*H 4·8 (see Note 3) | 200 ml. |

Both these solutions will keep for many months in hard glass glass-stoppered bottles; the stoppers should be covered with small beakers to discourage the entry of moulds. The working solution is made by mixing equal volumes of solutions A and B; it will keep for about a fortnight in a Coplin jar.

*Dehydrating and clearing fluids*

Acetone (in a drop bottle)
Acetone-xylene equal parts (in a glass-stoppered jar)
Acetone 10 per cent., xylene 90 per cent., by volume (in a Coplin jar)
Xylene I
Xylene II

*Mountant*

D.P.X. (Kirkpatrick and Lendrum, 1941). We can no longer obtain "Distrene 80". "Lustron, crystal clear, L 2020" (Monsanto Co.) is a suitable substitute.

## *Procedure*

1. Fix thin pieces of tissue as fresh as possible (see Note 4), for three days or longer. Dehydrate in graded alcohols, clear in cedarwood oil, rinse in xylene, imbed in paraffin.

2 Cut sections 3 5 $\mu$ thick, take down to water in the usual way, and blot

3 Stain in the working solution 10 minutes to 24 hours—the exact time makes little difference

4 Rinse in running distilled water for a few seconds A little of the pyronin is removed at this stage, and the exact time for perfect results should be found by trial Our routine time is seven seconds Blot nearly dry

5 Dehydrate quickly with acetone, rinse rapidly in acetone xylene followed by 10 per cent acetone in xylene and two changes of xylene Mount in D P X

### *Results*

Nuclei, mast cell granules (occasionally) green or blue green

Mucus green

Muscle, epithelial cytoplasm, nucleoli, young myelocyte granules pink or pinkish orange

Erythrocytes, granules of polymorphs and eosinophils, Paneth cell granules orange

Keratin, collagen glia fibres, necrotic material paler orange

Cytoplasm of lymphocyte and plasma cell series deep magenta

Cytoplasm of erythroblasts, basophil cytoplasm generally (*e g* Nissl substance, pancreatic acinar cells, hepatic parenchyma), bacteria shades of magenta

Mast cell granules (usually) very dense red brown to (almost) black

The method is particularly useful in the study of lymph nodes, bone marrow, inflammatory reactions and tumours

### *Notes*

1 Not all dyes sold as pyronin are suitable Some samples precipitate orange G We test samples as follows —

In a series of tubes, 0 3 per cent solutions of the pyronin are made in M/5 acetate buffers of *p*H 3 6, 4 2, 4 8 and 5 2 An equal volume of 0 1 per cent aqueous solution of orange G is added to each tube With a satisfactory pyronin, no precipitate should form in the *p*H 4 8 and 5 2 tubes , at *p*H 3 6 there may be a slight precipitate and at *p*H 4 2 a precipitate may form during twenty four hours, but not immediately More precipitation than this indicates that the pyronin is unsuitable We have found satisfactory a sample from G T Gurr, labelled " Pyronin, batch 1540 ", obtained in October 1950 This is of a bluish shade , it was sold as " Pyronin Y " Samples of " Pyronin Y " from other sources were a more yellowish red colour and precipitated orange G heavily

2 The methyl green must be heptamethylpararosaniline (salt) C I no 684 , it must be freed from methyl violets by repeated extraction of a 2 per cent aqueous solution with several changes of chloroform in a separating funnel, until the chloroform layer remains colourless We have called the resulting supernatant " 2 per cent methyl green ", although it contains less dye than this by the amount of violets extracted At present we are using " methyl green C I 684, batch 150, G T Gurr "

It has been stated that methyl green readily decomposes to yield methyl violets (Conn, 1946) We have found that a 2 per cent solution from which the violets have been extracted can be kept for six months without yielding sufficient methyl violet to colour chloroform used to extract it

We have some samples labelled " methyl green " which are soluble in chloroform and are useless for the present purpose One of these is spectro photometrically similar to malachite green, which is no substitute for methyl green in our method

3 We make the acetate buffer by titrating M/5 sodium acetate solution, with 6 5 per cent aqueous acetic acid solution, to *p*H 4 80+0 02, measured with a glass electrode A variation of 0 05 of a *p*H unit either way alters the staining ,

higher $p$H levels give more of the green stain, lower levels, more of the red. This can be corrected to some extent by altering the proportion of pyronin to methyl green; but at higher $p$H levels orange staining is fainter, and at lower levels orange G is precipitated by pyronin. However, we found that at $p$H 5·6, for example, the effect of small deviations in $p$H or in the proportion between the basic dyes is very much less than at $p$H 4·8; and although at the lower $p$H collagen is stained only faintly, polymorph and eosinophil leucocyte granules and erythrocytes are satisfactorily shown if extra orange G is used and there is less tendency to "blueing" of the nuclei with pyronin overlaying the green stain. The higher the $p$H, the less stable is the solution; methyl green is converted to a colourless soluble base, and pyronin to a coloured insoluble base or to its insoluble salt with orange G. The table shows what we have found to be the correct proportions of our dye samples, in staining mixtures made at different $p$H levels. As pyronin, in particular, varies greatly in dye content, it may be necessary to find the correct proportions for other samples by trial, The concentration of buffer is not so critical.

TABLE

*Amounts of dye to be added to* 100 *ml. of M*/10 *acetate buffer*

| $p$H of buffer | Pyronin (ml. of 5 per cent.) | Methyl green (ml. of 2 per cent.) | Orange G (ml. of 1 per cent.) |
|---|---|---|---|
| 4·2 | 3·8 | 5·0 | (precipitates) |
| 4·6 | 3·8 | 3·5 | 5·0 |
| 4·8 | 4·3 | 2·5 | 6·0 |
| 5·2 | 7·0 | 2·5 | 9·0 |
| 5·6 | 10·0 | 2·5 | 12·0 |
| 6·3 | 13·8 | 2·5 | 30·0 |

4. If the tissues are not fresh when fixed, or are fixed in acid formalin (below $p$H 4), the green nuclear stain is replaced or overlaid with pyronin staining. It has been stated (Kurnick, 1949-50; Kurnick and Mirsky, 1949-50) that methyl green combines with deoxyribonucleic acid only in its natural, polymerised form, and that pyronin stains ribonucleic acid and depolymerised deoxyribonucleic acid. It may be that autolysis, or acid fixation, depolymerises deoxyribonucleic acid. We have found that material that has been well fixed in "neutralised" formalin can be subjected to acid solutions (*e.g.* decalcifying agents) without interference with subsequent methyl green staining of nuclei. Mucus is stained green even in material fixed in acid solution.

## *Summary*

A method is given for the staining of paraffin sections of formalin-fixed material with a methyl green-pyronin mixture to which orange G has been added to stain acidophil elements. It is particularly useful in the study of lymph nodes, bone marrow, inflammatory reactions, and tumours.

We should like to acknowledge our debt to Dr C. L. Oakley for assistance in the preparation of this paper.

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# RAPID TENTATIVE POST-MORTEM DIAGNOSIS OF INFLUENZA *WITH THE AID OF CYTOLOGICAL SMEARS OF THE TRACHEAL* EPITHELIUM

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(Plates XLVIII and XLIX)

Since 1918 evidence has been accumulating that human influenza may be recognised *post mortem* by the fact that the ciliated and goblet cells of the tracheal and bronchial mucosa are destroyed by the virus, while the basal layer of epithelial cells is preserved. For the literature of this subject see Straub and Mulder (1948) and Mulder and Verdonk (1949). The question now arises whether it would be possible for the pathologist at a post-mortem examination to suggest from the examination of cytological smears from the tracheal mucosa the probability that the case in question is one of influenzal virus infection.

During the epidemic of influenza A-prime in Holland in January and February 1949 (van Bruggen *et al.*, 1950), we had the opportunity of investigating this point at 8 post-mortem examinations. One patient (case 3 in the table, p. 331) came from the psychiatric clinic, "Endegeest", and the lungs and a portion of the trachea of case 6 were kindly sent to us from Groningen by Drs. C. van Oven and T. G. van Rijssel.

## *Technique*

All patients who died with acute bronchial or pulmonary symptoms during the course of the epidemic were submitted to autopsy as soon as possible and in every case within 4 hours of death. A tracheal cylinder about ⅜ in. in length was excised just above the bifurcation with a very sharp sterilised knife. A sterile loop was then passed over the surface of the mucous membrane and from this layer a wet methylene-blue preparation was made. Other smears were stained, after drying, with May-Grunwald-Giemsa. Cultures on blood-agar and Levinthal-agar were made from the same portion of the trachea, which was then preserved at a temperature of −130° C. The remainder of the trachea was fixed *in toto* in formalin without being opened or washed out and histological sections were made from the part contiguous to the excised cylinder. In all cases, after clamping the arteries, the heart and lungs were removed, also

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*in toto*, and some blood was taken from the heart to be tested for antibodies against influenza virus. This test may show at what period of the influenzal attack the patient died, because the clinical diagnosis was not always made during life and no blood serum was available in the clinic for immunological investigations.

### *Cytological picture of normal tracheal epithelium in methylene-blue-stained smears*

This consists of three different kinds of cells :—(1) ciliated epithelium, (2) goblet cells and (3) basal cells. All three can be easily recognised (figs. 1-4).

### *Virological and serological examination for influenza*

From none of the patients were any garglings or sputa available for virological examination because all were very ill on admission. Two died on the day after admission, the others after only a few hours. No clinical data were available for case 3 and no blood serum for serological examination was available from 2 of the 8 cases. The cylindrical portion of the trachea was cut open after being thawed and the mucous membrane scraped off with a sharp spoon. This was ground with sterile sand in 10 ml. of phosphate buffer (*p*H 7·0), to which were added penicillin (500 units/ml.), streptomycin (500 units/ml.) and sulphamethylpyrimidine (200 mg. per 100 ml.). The emulsion was inoculated into the amnion of ten 13-day-old chick embryos. After three days' incubation at a temperature of 35° C. the amniotic fluid was examined for influenza virus. Two amniotic passages were performed if no virus was found in the first fluids. In the course of the epidemic, experience showed that in the positive cases the first amniotic inoculation usually contained virus and the second passage did so in every instance. It is reasonable to assume, therefore, that the obtaining of two negative amniotic passages clearly indicates that no influenza A virus is present, though influenza B might be. All strains isolated were classified serologically with antisera of ferrets against influenza B, strain Lee (U.S.A., 1940), swine influenza, Shope 15 (U.S.A., 1940), A-WS (England, 1933), A-PR8 (U.S.A., 1933), A (Holland, 1941), A-prime Rhodes (U.S.A., 1947), FM1 (U.S.A., 1947), or Heer (Holland, 1949). All isolated strains clearly belonged to the A-prime group (van der Veen and Mulder, 1950). Portions of the inflamed lungs were examined in the same way to find out if they contained influenza virus.

In our opinion the significance of demonstrating influenza virus in trachea and lungs has not been ascertained. Since we found that garglings may contain an influenza-virus concentration of $10^{-7}$ per ml., it is clear that virus, which may have been aspirated *ante mortem*, can be isolated from the respiratory tract and the lungs. Quantitative examinations may perhaps shed light on this question, until methods have been devised which will demonstrate the virus *in situ* in the cells of the respiratory tract, including the lungs.

The blood serum was titrated for antibodies with the aid of the hæmagglutination-inhibition test against the strains A-PR8, A-prime Rhodes and Heer. The complement-fixation test was performed against A-PR8 or A-prime Rhodes and against influenza B Lee. With one exception all titres were very low, so that it was evident that the patients with a positive virus isolation had died within 5 or 6 days of the onset of the disease. In one case (no. 8) the blood serum showed inhibiting titres of 4100 against the strain PR8, 3550 against the A-prime strain Rhodes and 2300 against the A-prime strain Heer. In the complement-fixation test the titres were 10240 against PR8 and 40 against Lee (the lowest dilution of the serum). The positive titres are so high that they may be taken as proof of a recent infection with influenza A (A-prime).

CYTOLOGICAL DIAGNOSIS OF INFLUENZA

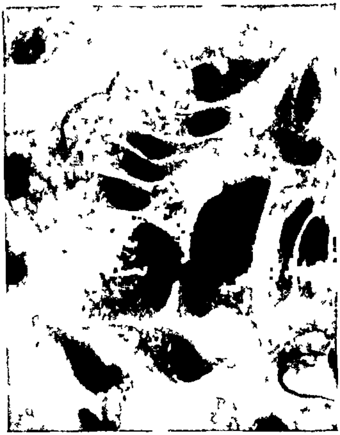

FIG 1 —Case 1 Control Cytological smear from trachea Normal epithelium with goblet cells, but ciliation not visible in this area Methylene blue ×1100

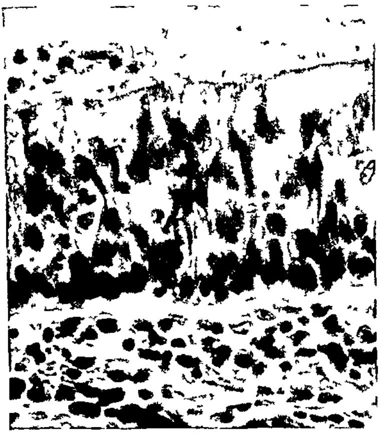

FIG 2 —Case 1 Control Corresponding section of trachea Normal ciliated epithelium Clear signs of inflammation ×600

FIG 3 —Case 2 Control Cytological smear from trachea, showing normal ciliated epithelium Methylene blue ×1200

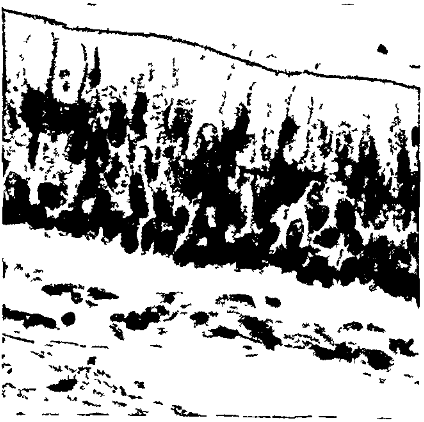

FIG 4 —Case 2 Control Corresponding section of trachea Normal ciliated epithelium with goblet cells No sign of inflammation ×600

TABLE—*Survey of six cases of influenzal pneumonia with epithelial lesions in the respiratory tract and two control cases (nos. 1 and 2)*

| Case no | Sex and age | Duration of illness (days) | Bacteriology of trachea and lungs | Antibodies against influenza A and B | Virus isolations from trachea or lungs or both | Post mortem diagnosis | Cytology of trachea | Epithelium in corresponding section of trachea | Epithelium at other levels of trachea and bronchial tree |
|---|---|---|---|---|---|---|---|---|---|
| 1 | F 70 | 7 | *Hæmophilus influenzæ* | Negative | Negative | Myocardial infarction Slight tracheitis Capillary bronchitis | Normal ciliated epithelium | Normal ciliated epithelium | Normal ciliated epithelium |
| 2 | F 76 | 8 | Pneumococcus type III | Negative | Negative | No tracheitis Lobar pneumonia in left upper lobe | Normal ciliated epithelium | Normal ciliated epithelium | Normal ciliated epithelium |
| 3 | F 69 | 3 | *Hæmophilus influenzæ* and Pneumococcus type III | Negative | Positive (A prime) | No tracheitis Bronchopneumonia in both lower lobes and right middle lobe | No ciliated epithelium | 2 or 3 layers of undifferentiated epithelium with necrobiosis | Locally well preserved At other places undifferentiated epithelium—sometimes 2 or 3 layers |
| 4 | F. 40 | 3 | *Staphylococcus aureus* | Negative | Positive (A prime) | Fibrinous necrotising tracheitis Diffuse bronchopneumonia in both lower lobes and right middle lobe | No ciliated epithelium | 2 or 3 layers of undifferentiated epithelium with mitoses | Undifferentiated epithelium |
| 5 | F 60 | 5 | *Staphylococcus aureus* | Negative | Positive (A prime) | Fibrinous necrotising tracheitis Diffuse bronchopneumonia in both lower lobes and right middle lobe | No ciliated epithelium | 5 or 6 layers of undifferentiated epithelium with mitoses | Undifferentiated epithelium |
| 6 | M 16 | 2 | *Staphylococcus aureus* |  | Positive (A-prime) | Fibrinous necrotising tracheitis Confluent bronchopneumonia in all lobes of both lungs | No ciliated epithelium | 1 or 2 layers of undifferentiated epithelium with mitoses | Undifferentiated epithelium |
| 7 | F 84 | 5 | Chains of streptococci in smears (culture negative) | . | Positive (A prime) | No tracheitis Bronchopneumonia in both lower lobes and right middle lobe | Ciliated epithelium partly degenerated | For the most part normal ciliated epithelium A few areas with 2 and 3 layers of undifferentiated epithelium | Large areas of undifferentiated epithelium with 1 and 2 layers |
| 8 | F 54 | 14 | Chains of streptococci in smears (culture negative) | Very high against A | Negative | Cirrhosis of liver and hemorrhage from œsophagus No tracheitis Focus of pneumonia in left lower lobe | Ciliated epithelium partly degenerated | Normal ciliated epithelium A single area with 4 and 5 layers of undifferentiated epithelium | Large areas of regenerating undifferentiated epithelium besides areas with normal ciliated epithelium |

### *Results of the examination*

From the data in the table it may be seen that 6 out of the 8 cases analysed were due to an infection with influenza virus and that 7 of the patients were women. Patient no. 8 suffered from cirrhosis of the liver and died of hæmorrhage from an œsophageal varix. There was a large focus of purulent inflammation in the dorsal segment of the left lower lobe. Cases 4, 5 and 6 showed extensive fibrino-purulent tracheo-bronchitis caused by *Staphylococcus aureus*. This was absent in cases 3, 7 and 8. Cases 7 and 8 showed a slight, probably bacterial, tracheitis. Case 3 showed no signs of bacterial inflammation in the tracheal mucosa. Cases 3 (figs. 5 and 6), 4, 5 (figs. 7 and 8) and 6 could easily be identified as influenza from the cytological smears, since the ciliated epithelium was either entirely absent or showed distinct necrobiosis. In cases 7 and 8 judgment was more difficult, as the ciliated epithelium was not entirely absent, but showed distinct degeneration in places. Sections of the trachea also showed areas of local epithelial degeneration. The ciliated epithelium at higher levels of the trachea in case 7 and of the bronchi in case 8, however, was unmistakably destroyed and showed regenerating undifferentiated epithelium. The cytological smear of case 5 showed mitosis of the basal cells (figs. 7 and 8).

It is evident that cytological examination of the trachea and bronchi by the smear method in cases like these can never yield any certainty as to the existence of an influenzal infection. To the pathologist and the virologist, however, they may indicate the possibility of the existence of an infection with epitheliotropic virus in the respiratory tract. As such, they may be useful in suggesting to the virologist the desirability of undertaking a search for virus in the mucosa and to the pathologist of making a careful histological study of the epithelium of trachea and bronchi. Further detailed cytological studies will probably shed more light on the finer structural changes which occur in the degenerations and regenerations of the respiratory tract epithelium in human influenza.

### *Summary*

The authors are of the opinion that it may often be possible to suggest the existence of an influenzal infection in the course of a post-mortem examination by making an immediate cytological examination of the epithelium of the tracheal and bronchial mucosa.

An influenzal viral infection is indicated if smears from the tracheal mucosa show complete absence or great reduction in number of ciliated epithelial cells and their replacement in whole or in part by undifferentiated and necrobiotic epithelium.

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CYTOLOGICAL DIAGNOSIS OF INFLUENZA

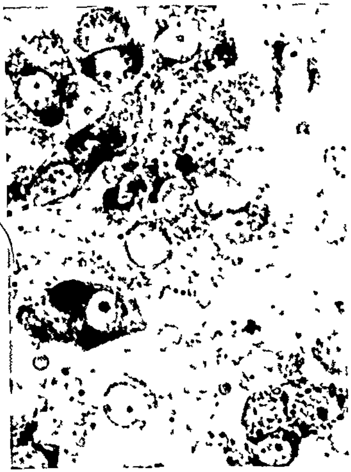

FIG 5 —Case 3 Cytological smear of trachea No ciliated cells Severe degeneration with hyperchromatism of nuclear wall Methylene blue ×1200

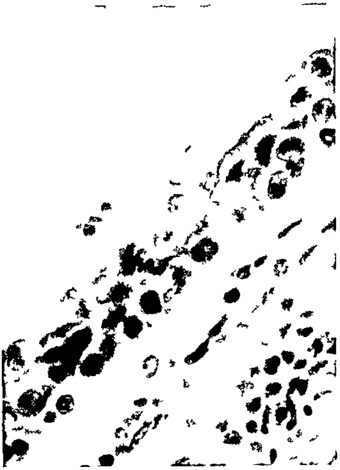

FIG 6 —Case 3 Corresponding section of trachea No ciliated epithelium The basal layer of epithelial cells is preserved Distinct necrobiosis with swollen cells and nuclei some of them with hyperchromatism of the nuclear wall ×600

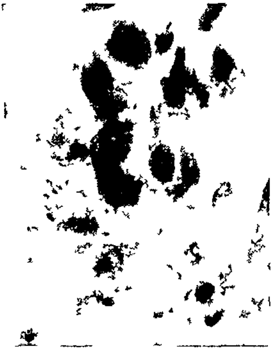

FIG 7 —Case 5 Cytological smear of the trachea No ciliated epithelium Two mitoses Methylene blue ×1200

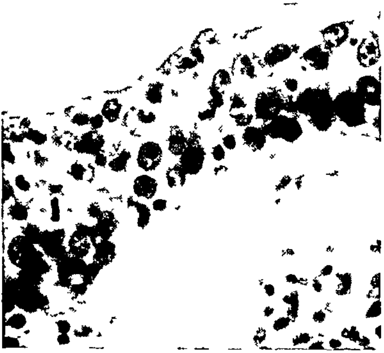

FIG 8 —Case 5 Corresponding section of trachea Regeneration from basal cells to 4 or 5 layers of undifferentiated epithelium Swelling of cells and nuclei and hyperchromatism of nuclear wall Mitoses in a few cells ×600

576 . 8 . 093 . 1 : 576 . 852 . 215 (*M. johnei*)

# VARIETIES OF *MYCOBACTERIUM JOHNEI* ISOLATED FROM SHEEP

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Taylor (1945) confirmed the implication in the work of earlier authors that there are two diseases of sheep simulating Johne's disease (paratuberculosis) of cattle. The first of these, true Johne's disease of sheep, has been recognised for many years and is known in Europe, India, and America; it is caused by the classical type of *Myco. johnei*, identical with the organism isolated from cattle. The second condition is widespread in Scotland and is characterised by the yellow or orange colour of the affected intestine and the enormous number of acid-alcohol fast bacilli present in the lesions. Attempts to isolate the organism responsible for this form of the disease have consistently failed and cultures have not so far been described. Johne's disease of sheep in Iceland is another example of a similar condition caused by an organism resistant to artificial cultivation, for although the disease has been the subject of several recent papers (Sigurdsson and Tryggvadóttir, 1949) cultures have never been obtained (Palsson, personal communication; Glover, personal communication). This paper describes the isolation in culture of strains of acid-alcohol fast organisms resembling *Myco. johnei* from sheep in Scotland and Iceland.

## *Materials and methods*

From 1948-50, fifteen specimens of intestine from different sheep affected with Johne's disease were received. One from Edinburgh was of the non-pigmented type; four with no unusual colouring came from Iceland; and ten showing the orange-coloured type of lesion came from Aberdeen and the north of Scotland.

Mucous membrane from each piece of intestine was treated with oxalic acid to destroy contamination before inoculation on a number of different media, which included some of the following: Dunkin's medium for *Myco. johnei* but without crystal violet; the American Trudeau Society's egg-yolk potato medium; a slightly modified Finlayson medium (Taylor, 1950) and Jamieson's egg-yolk agar. All of these contained 4 per cent. of glycerine and approximately 1 per cent. of heat-killed *Myco. phlei*. In addition, Finlayson's medium both with and without 4 per cent. of glycerine was used.

## *Results*

The classical bovine type of *Myco. johnei* was isolated from one of the fifteen specimens examined: that received from Edinburgh. In primary culture it grew readily after four weeks' incubation on Dunkin's medium, modified Finlayson medium and the American Trudeau Society's medium. Culturally and microscopically the growth was typical of *Myco. johnei*. There was no growth on media without killed *Myco. phlei*.

Each of the four specimens from Iceland yielded a culture morphologically similar to *Myco. johnei*, but slower to develop. In primary culture no growth was apparent until the seventh week of incubation. It appeared on modified Finlayson's medium as very small whitish colonies which did not increase greatly in size on further incubation. Growth never resulted on Dunkin's medium either in primary culture or on first subculture, and profuse growth was not obtained on any medium even after the sixth subculture. Media without killed *Myco. phlei* do not support growth.

From each of the ten specimens of the orange-coloured type of lesion sent from the north of Scotland, highly pigmented strongly acid-alcohol fast bacilli were isolated. These grew well in primary culture on modified Finlayson medium after incubation for from 6-8 weeks and nearly as well on good batches of American Trudeau Society's medium ; but growth was not obtained on Dunkin's medium or Jamieson's yolk agar even after continuous incubation for periods of up to twelve months, nor has it occurred on media without the addition of killed *Myco. phlei.* Single colonies were smooth, convex, and glistening; the growth was soft, readily emulsified and of a bright orange colour. Secondary colonies, such as occur frequently with *Myco. johnei,* were not observed. The colour was present from the time of visible growth, developed in the incubator, and was affected neither by prolonged incubation nor exposure to light at room temperature.

It was apparent that both the Icelandic and the pigmented strains differed from *Myco. johnei* in at least one of their cultural requirements in that neither would grow in primary culture on a medium containing a low proportion of egg yolk : Dunkin's medium contains 15-20 per cent. of yolk and the modified Finlayson medium, 60 per cent. To test the value of egg yolk in supporting growth, therefore, media were prepared in which the proportion of egg yolk ranged from 15-60 per cent. : each medium contained in addition 4 per cent. of glycerin and 1 per cent. of heat-killed *Myco. phlei.* For the purposes of experiment, it was assumed that the egg white in the medium was inert. These media were inoculated with equal quantities of dilute suspensions of culture in Ringer solution and incubated for three months. The results in the table indicate that growth of either the Icelandic or the pigmented types in primary culture is unlikely on an egg medium containing less than 50 per cent. of yolk, whereas the classical bovine strains are less fastidious and can be grown on media containing only 25 per cent. of yolk.

TABLE

*The growth of varieties of* Myco. johnei *on egg media with different proportions of yolk and white*

| Inoculum | Growth in media with egg yolk (Y) and egg white (W) in the amounts shown (per cent.) | | | | |
|---|---|---|---|---|---|
| | Y = 15<br>W = 60 | Y = 25<br>W = 50 | Y = 37<br>W = 37 | Y = 50<br>W = 25 | Y = 60<br>W = 15 |
| *Myco. johnei* (bovine) primary culture | − | + | + | + | + |
| Icelandic strain, 6th subculture . | − | − | + | + | + |
| Pigmented strain, 6th subculture . | − | − | + | + | + |
| Pigmented strain, 3rd subculture . | − | − | − | + | + |
| Pigmented strain, 2nd subculture . | − | − | − | + | + |

− = no growth    + = growth

To compare the pathogenicity of the organisms for laboratory animals with that of *Myco. johnei* three pigmented and one Icelandic strain were each inoculated separately into two guinea-pigs intraperitoneally and one rabbit intravenously. One bovine strain was inoculated similarly as a control. The cultures were grown on modified Finlayson medium for 8 weeks, suspended in Ringer solution and inoculated in a dose of 10·0 mg. The animals were tested intradermally with 0·1 ml. of both avian and mammalian P.P.D. tuberculins (Ministry of Agriculture) at three and again at seven weeks after inoculation, when they were destroyed for examination. In rabbits no response to either tuberculin was produced by any of the strains and in guinea pigs, although the response was poor, the reaction to avian tuberculin was in each case greater than to mammalian The result of the autopsy on each animal was similar to that to be expected after the inoculation of a large dose of *Myco johnei* or some other Mycobacterium not normally considered pathogenic for laboratory animals All were in good bodily condition and lesions were not found except in one guinea pig, which showed one slightly enlarged and caseous mesenteric lymph node Cultures were prepared from the liver of the rabbits and the spleens and mesenteric lymph nodes of the guinea pigs Organisms were grown from the liver of two of the five rabbits, from the lymph nodes of all the guinea pigs, and from four of the ten guinea pig spleens, but in each case the number of colonies was small, giving no suggestion of a progressive infection The pigmented strains showed no alteration in morphology or colouration after passage through the guinea pig

## *Discussion*

The isolation in culture of two varieties of Johne's bacilli hitherto considered resistant to cultivation *in vitro* was accomplished by the use of a medium superior to that formerly employed in work of this nature and provides another example of the value of egg yolk as a major constituent of media used for the primary isolation of mycobacteria

It is probable that neither the Icelandic nor the pigmented strains are other than varieties of the classical type of *Myco johnei* The three organisms are the apparent causes of what is, both clinically and pathologically, essentially the same disease in sheep and cattle The new strains have a uniformly low virulence for laboratory animals Further, the two varieties share with *Myco johnei* its unique requirement of an accessory factor in the form of killed acid fast organisms in the medium upon which it is grown

Fundamentally the Icelandic strains appear to differ but little *in vitro* from *Myco johnei* The difference lies in their inability to grow on a medium which will support the classical type, and their slightly slower rate of growth On the other hand the ten pigmented strains probably form a definite type or variety of *Myco johnei*, and the distinction "ovine type" has already been suggested (Taylor, 1945) Their relationship with *Myco johnei* has been described in this paper, but the brilliant colour of the growth and the morphology and consistency of the colonies are all distinct from those of the classical type It is well known that the Mycobacteria as a genus have a tendency towards pigmentation, but this is not common among the pathogenic species except under certain well defined conditions The pigmentation of these cultures, however, appears to be an inherent character as it is present at all stages of growth and is not altered by animal passage Indeed the orange colour of the lesions of the disease in sheep, which was said by Taylor (1945) to be due to staining with bile, is in all probability caused by the colour of the organisms themselves as they are present in the lesions in very large numbers

## *Summary*

Johne's disease (paratuberculosis) of sheep may be caused by one of three related organisms (1) *M johnei*, the classical bovine type, (2) a similar organism isolated from sheep in Iceland, and (3) a highly pigmented organism isolated from sheep in Scotland

Growth of the Icelandic and pigmented strains, hitherto said to be resistant to artificial cultivation, is described for the first time, both are probably varieties of the classical type

I am indebted to Dr S. Jamieson, Aberdeen, Dr P. A. Palsson, Reykjavik, and Mr J. T. Stamp, Edinburgh, who provided the original material which made this work possible.

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616 . 36—006 . 326

# A CASE OF LIPOMA OF THE LIVER

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(Plate L)

References in the literature to lipomas of the liver are scanty. Weiss (1935) questions whether one has ever actually been found, while Rolleston and McNee (1929) state that " Genuine fatty tumours do not occur in the liver ".

## *Case report*

*Clinical history.* The patient, a female aged 54, was known to be a diabetic and to have severe hypertension (B.P. 230/110) about 18 months before admission. The diabetic symptoms were controlled by diet. She was admitted to Westminster Hospital on 13.3.49 on account of precordial pain of 24 hours' duration. On examination, a pericardial friction rub was heard. B.P. was 150/90. The liver was palpable four fingers' breadth below the costal margin. Urine was of low specific gravity, and contained much albumin and a trace of blood, but no sugar. A diagnosis of decompensated hypertension with renal failure was made. She developed auricular fibrillation and began to vomit. The vomiting became more severe, and contained " coffee ground " material. She became progressively weaker and died 12 days after admission.

*Autopsy findings.* The body was that of a very obese elderly female subject. The pleural sacs were dry. The lungs were firm and somewhat congested. The pericardial sac contained about half a pint of blood-stained fluid, and both layers of the pericardium were greatly thickened and roughened by recent fibrinous pericarditis. The heart showed gross left ventricular hypertrophy and, with the pericardium, weighed 33 ounces.

The gall bladder was small and fibrotic and contained pigment stones and muco-pus. Externally the liver was normal. On section, a round yellow nodule about ¾ in. in diameter was found in the right lobe (fig. 1). The nodule was well demarcated without appearing to be encapsulated, had a smooth, glistening cut surface and looked like fat.

The pancreas was fibrotic. The kidneys were pale and of normal size, with a finely granular surface on which small telangiectatic areas could be seen. On section there was some blurring of the cortico-medullary junction, and there was necrosis of one of the renal pyramids. The other organs were normal.

Death was due to renal failure and pericarditis.

*Histology.* The nodule in the liver had the typical appearances of adipose tissue. In the centre, the fat spaces of which it was largely composed were

LIPOMA OF THE LIVER

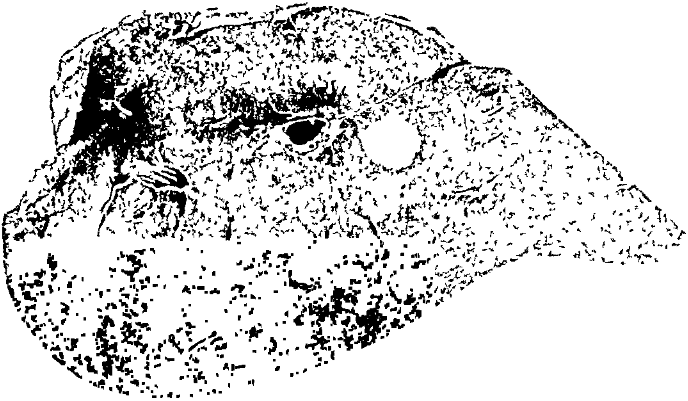

FIG. 1 —Lipoma in the right lobe of the liver. ×0 625

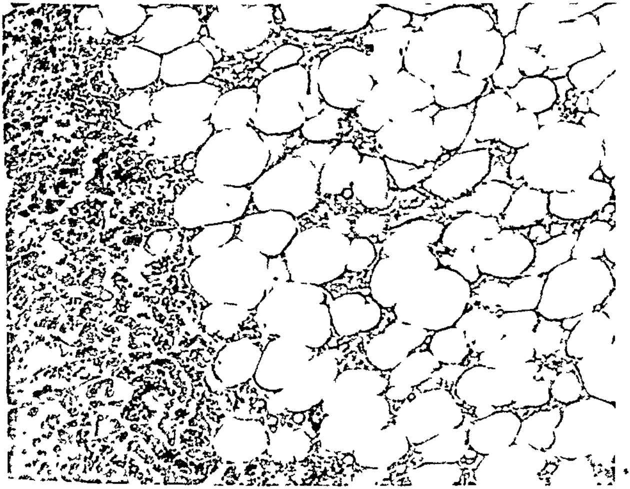

FIG 2.—Photomicrograph, showing margin of the lipoma
Heidenhain's azo carmine ×135.

between 60 and 90 $\mu$ in diameter. The stroma consisted of loose connective tissue containing oval or flat pale-staining nuclei. Blood vessels were small and not very numerous.

Near the margin, small groups of atrophic liver cells were incorporated in the growth. The stroma was somewhat more cellular and smaller fat spaces measuring as little as 10 $\mu$ were seen (fig. 2).

The margin was rather irregular, and there was no capsule. There was no distortion of nearby liver architecture by pressure but occasional individual liver cells appeared to be slightly compressed. A few of the liver cells nearest to the tumour possessed fat vacuoles.

Elsewhere the liver was normal, and there was no evidence of fatty degeneration or infiltration.

## *Discussion*

While it appears that lipomas of the liver are something of a rarity, it is said that they may be simulated in several ways.

Rolleston and McNee describe small nodules of adipose tissue in the liver caused by detachment of appendices epiploicæ from the colon with subsequent lodgment between the liver and the diaphragm. Such nodules are situated near the periphery of the liver and are surrounded by invaginations of the liver capsule. They also state that localised areas of extreme fatty infiltration, usually wedge-shaped, are not uncommon. They do not, however, mention whether generalised fatty infiltration of the liver is also present in such cases. Lichtman (1949) states that liver-cell adenomata, multiple, light brown, or greyish white, situated in the midst of normal liver and with their large cells laden with fat or glycogen, may resemble lipomas.

In the case described the nodule lay a considerable distance from the surface of the liver and was not encapsulated. The fat spaces were enormously greater in size than those found in fatty infiltration, and fatty infiltration was not present elsewhere in the liver. There was no evidence of adenomatous arrangement. The appearances of the nodule, both macroscopic and microscopic, were those of adipose tissue. None of the patient's symptoms appear to be related to it, and its discovery at autopsy was entirely accidental.

## *Summary*

A case is described in which a round, yellow nodule with the features of adipose tissue, was found in the liver at autopsy. Differential diagnosis is briefly discussed.

My thanks are due to the Department of Medical Photography of Westminster Hospital.

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616—006—018.4

# OSSIFICATION IN INTESTINAL NEOPLASMS: A REPORT OF THREE CASES

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(Plate LI)

While heterotopic ossification in various tissues has frequently been described in man and animals (Keith, 1927-28), it has rarely been seen in intestinal neoplasms (Clark, 1934-35; Dukes, 1938-39; Scheidegger, 1939). The three cases described below show the formation of membrane bone in the primary growth and in recurrences and metastases.

## *Case reports*

**Case 1.** V. L., a female aged 44, was admitted to the Royal Cancer Hospital in September 1949 complaining of pain in the abdomen and back, intermittent passage of bright red blood in the stools, and loss of weight for the last twelve months. On examination an enlarged, lumpy liver and a carcinoma of the rectum, the latter confirmed histologically, were found. As a palliative operation, removal of the rectum, uterus, Fallopian tubes and ovaries was undertaken. About 8·5 cm. from the ano-rectal junction, the rectum contained a large, ulcerated, fungating carcinoma, 7·5 cm. long, which completely encircled the lumen and reduced it to about 1·0 cm. in diameter. Posteriorly, the neoplasm had infiltrated for a distance of 3·5 cm. beyond the muscle coat and anteriorly it had broken through the rectal wall to form a mass in the recto-vaginal septum binding it to the cervix. At several places in the rectal wall there were subserosal nodules of growth. On section the neoplasm posterior to the rectum contained widespread spicules of osseous tissue. Several adjacent lymph nodes were replaced by metastatic growth. Massive ascites developed in March 1950, and the patient died four months later.

*Histological findings.* The tumour is a well-differentiated tubulo-papillary adenocarcinoma, necrotic in parts, with a dense fibrocellular connective tissue stroma containing spicules of bone formed by intra-membranous ossification. These spicules are, for the most part, surrounded by a continuous layer of plump osteoblasts laying down osteoid matrix. Some of the bone is necrotic. The loose connective tissue between the groups of bony spicules contains numerous dilated capillaries lined by endothelium only, as in bone marrow.

**Case 2.** In December 1943, F. W., a male aged 48, visited the New York General Hospital complaining of vomiting and abdominal pain. An X-ray of the bowel revealed nothing abnormal. In August 1944, he was operated on for intestinal obstruction. His symptoms recurred in February 1945, and, at operation, adhesions were freed. A further laparotomy at the Royal Cancer Hospital in December 1945, disclosed multiple tumour deposits in the peritoneal cavity. He died in January 1946.

At autopsy the bowel contained a tumour (exact site not specified) with a gritty surface to which several coils of intestine were adherent. A large secondary deposit in the right rectus abdominis muscle extended from the costal margin to a point three inches above the symphysis pubis.

*Histological findings.* The tumour is an adenocarcinoma in a dense fibrocellular connective tissue stroma and partly surrounded by necrotic skeletal muscle fibres, many of which contain deposits of calcium. Within the stroma, in close relation to tumour cells, there are many spicules of bone, some of which

OSSIFICATION IN INTESTINAL NEOPLASMS

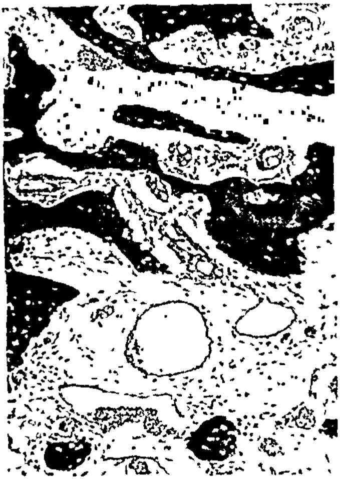

FIG. 1.—Spongy bone, in Case 2, showing both tumour acini and dilated vascular sinuses in marrow spaces. Van Gieson. 90

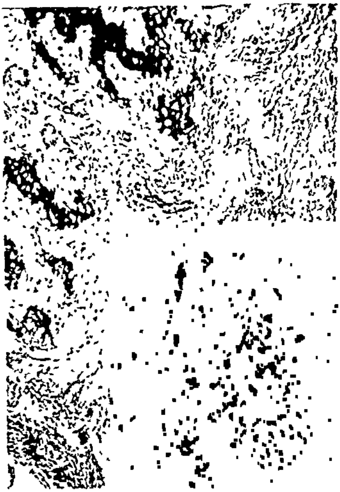

FIG. 2.—Another area of the stroma in Case 2, showing the transformation of connective tissue into bone with lacunæ containing bone corpuscles and, in right lower corner, the condensation of connective tissue to form a periosteum. Van Gieson. ×70.

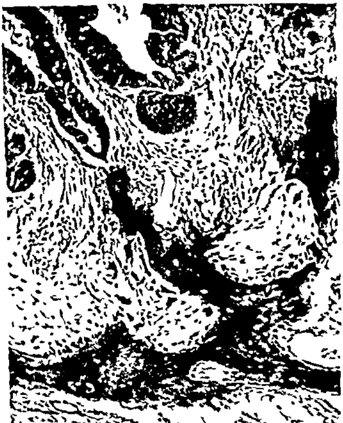

FIG. 3.—Stroma in Case 3, showing formation of bone surrounded by chains of plump osteoblasts. Picro Mallory. ×100.

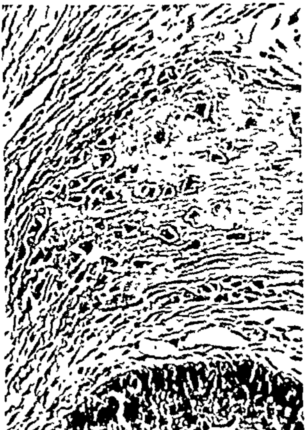

FIG. 4.—Case 3, showing transformation of connective tissue and fibrocytes into osteoid tissue and osteoblasts respectively. Picro-Mallory. ×160.

are grouped together into zones of spongy bone with loose fibrocellular connective tissue and dilated vascular sinuses like those of bone marrow within the interstices (figs. 1 and 2).

**Case 3.** M. T., a female aged 43, was admitted to another hospital in 1945 complaining of diarrhœa and intermittent bleeding per rectum for one year. A carcinoma of the rectum was removed by perineal excision. In June 1946 the patient was admitted to the Royal Cancer Hospital with an " abscess ", of nine weeks' duration, in the buttock. On examination, an ulcerated, hemispherical mass, approximately two inches in diameter, and continuous with a large fixed mass within the pelvis, was found behind the vulva. This mass, histologically an ossifying adenocarcinoma, was excised. Two further recurrences, both ossifying adenocarcinomata, were excised in November 1946 and January 1947. The last was a massive growth, measuring 10·0×7·5 cm., situated over the upper part of the perineal wound and adherent to the underlying sacrum. At operation only part of the tumour was removed. On this occasion the serum alkaline phosphatase was 21 King-Armstrong units per 100 ml. (normal range: 5-10 units). Death occurred in March 1947. Permission for an autopsy was not forthcoming.

*Histological findings.* All three recurrences (June 1946, November 1946 and January 1947) show rapidly-growing, tubulo-papillary adenocarcinomata with numerous spicules of bone within the fibro-cellular connective tissue stroma. There is active osteogenesis, with complete chains of plump osteoblasts surrounding the spicules and laying down osteoid tissue (figs. 3 and 4).

## *Discussion*

In all three cases ossification was intramembranous and had occurred in close proximity to living neoplastic epithelium. As described by Dukes, there were, in all the tumour deposits, whether primary, secondary or recurrent, areas of necrosis, but these were often sparse and usually not closely related to sites of ossification, and occasionally the bony spicules were themselves necrotic. The tumour stroma in each case was relatively avascular. The adenocarcinomatous epithelium showed no atypical histological features distinguishing it from the usual intestinal carcinomas. Dukes emphasised that in his cases there was both clinical and histological evidence of slow growth. This was not so in the 3 cases now reported; numbers 1 and 3 showed numerous mitotic figures indicating rapid growth, and the duration from the initial onset of symptoms to time of death was approximately 2, 2½ and 3 years respectively.

Scheidegger described a case of stroma ossification in a rectal carcinoma in which incidental autopsy findings included ossification in both a small mammary fibroadenoma and a leiomyoma of the stomach. This example of widespread ossification must be due to an individual constitutional idiosyncrasy. Clark reports a case similar to case 2 above in which an ossifying adenocarcinomatous metastasis developed in the laparotomy scar (right paramedian) within six months of operation, the primary growth being an inoperable carcinoma of the pelvic colon.

Where ossification occurs in intestinal neoplasms it is most likely that the tumour epithelium itself has the property of inducing osteogenesis. The actual method of formation of bone in heterotopic sites, such as operation scars and tumour stroma, has aroused considerable discussion amongst pathologists for many years. Although the whole mechanism is by no means understood as yet, it now seems beyond doubt that the osteoblasts develop *in situ* from certain cells (probably fibroblasts) in the connective tissue stroma. In all three cases described there are many areas in which cells can be seen in all stages of transition between long slender inactive fibrocytes and typical plump rounded or polygonal osteoblasts both within and covering the surface of the osteoid tissue. This is well shown in case 3 (fig. 4) which also clearly exhibits the transformation of connective tissue into hyaline eosinophilic material surrounded by osteoblasts, that is, into osteoid tissue. Once bone has appeared there is a condensation of the surrounding fibrous tissue to form a periosteum with osteoblasts between it and the osteoid tissue (fig. 2). Complete chains of plump osteoblasts laying down relatively broad zones of osteoid, as yet uncalcified, indicate active osteogenesis. The intervening connective tissue stroma within groups of spicules is loose and vascular, the vessels being, like those seen in bone marrow, dilated capillaries lined by a single layer of endothelium (fig. 1). It is interesting to note that this type of vessel is not present in the connective tissue stroma remote from areas of ossification.

It thus appears that the spongy bone forms first and then, once this has happened, the tissue within the interstices is transformed into bone marrow. This may sometimes develop hæmopoietic elements as in Lyall's (1943-44) case, but this did not obtain in the present 3 cases. Osteoclastic absorption was absent in all 3 cases.

## *Summary*

Three cases of stromal ossification in intestinal adenocarcinomata, two of which were rectal in origin, are described. In one a secondary growth in the right rectus abdominis muscle and in another the three recurrences were ossified.

Ossification was intramembranous. Where spongy bone developed typical marrow stroma appeared in the interstices although no immature erythrocytes or leucocytes were present.

I am grateful to Professor R. A. Willis for his helpful criticism ; to members of the Consultant staff for access to the clinical notes ; to Mr C. G. Chadwin for the sections and to Mr L. A. Cowles for the photomicrographs.

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616 . 71—018 . 46—006 . 442

# A CASE OF HISTIOCYTIC MEDULLARY RETICULOSIS

V. J. McGovern, A. W. Morrow and E. F. Thomson

*Royal Prince Alfred Hospital, Sydney*

(Plate LII)

Hypersplenism is a concept of functional hyperactivity of the spleen by which Doan and Wright (1946) seek to explain certain anæmias found in conditions associated with enlargement of the spleen. In view of the findings in the case presented here, together with those of Scott and Robb-Smith (1939), some modification of this concept must be made.

## *Clinical history*

The patient was a young woman aged 27 years. There was nothing remarkable in her previous history or in her family history.

HISTIOCYTIC MEDULLARY RETICULOSIS

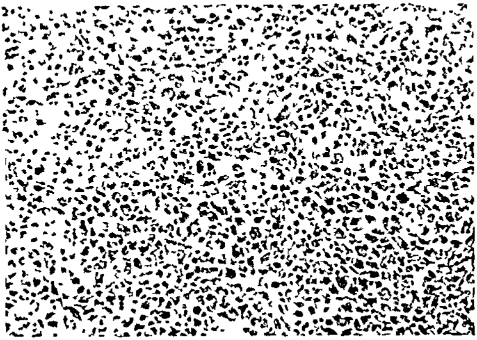

FIG. 1.—Spleen. The normal splenic architecture is obscured by a proliferation of erythrophagocytic histiocytes. Hæmatoxylin and eosin. ×100.

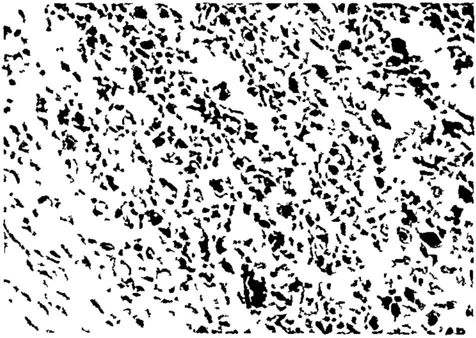

FIG. 2.—Spleen. Higher magnification showing erythrophagocytic histiocytes. H. and E. ×200.

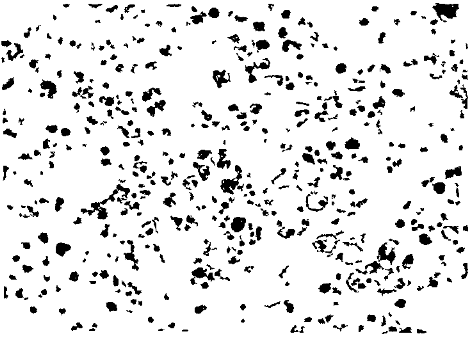

FIG. 3.—Sternal bone marrow showing hypoplasia of hæmopoietic tissue and slight increase in erythrophagocytic histiocytes. H. and E. ×200.

She was quite well until August 1949 when she commenced to suffer from a fever with some sweating and vague generalised pain The fever was intermittently present through August and September but during October became more apparent and the sweating became more drenching At this stage the nocturnal temperature was sometimes in the vicinity of 104° and 105° F, her pulse up to 130 per minute and respirations 20 per minute There were intermittent attacks of diarrhœa The provisional diagnosis of Brucellosis was made whereupon streptomycin and sulphadiazine treatments commenced This was without effect Blood counts revealed that she had developed a progressive anæmia and so she was given two litres of blood, one on 10/12/49 and one on 12/12/49 at Canberra Community Hospital She was admitted to Royal Prince Alfred Hospital, Sydney (16/12/49) at which time it was noted that there was slight enlargement of her spleen and a mild generalised lymphadenopathy The fever did not abate and drenching sweats were frequent and occasional episodes of diarrhœa were noted In December she commenced to complain of pains in the lower extremities particularly in the tibiæ and in the feet The enlargement of the spleen became more apparent Because of her failure to respond to all forms of antibiotic and chemotherapeutic treatments the provisional diagnosis of abdominal Hodgkin's disease was made, and deep X ray therapy was applied to the splenic area without any satisfactory result

She rapidly became more anæmic and terminally there was a generalised purpuric rash with some degree of icterus She died on 15 2 50

On admission to Royal Prince Alfred Hospital her blood count was as follows —Erythrocytes 3,870,000 per c mm, hæmoglobin 11 g per cent, reticulocytes 0 5 per cent, leucocytes 1300 per c mm, neutrophils band forms 12 per cent, segmented forms 52 per cent, total neutrophils 64 per cent, lymphocytes 18 per cent, monocytes 2 per cent, unclassifiable 2 per cent, platelets 21,000 per c mm The red blood cells showed poikilocytosis and anisocytosis of increasing degree in the course of the illness

On 6 2 50, the blood count was as follows —Erythrocytes 1,200,000 per c mm, hæmoglobin 4 g per cent, leucocytes 850 per c mm, neutrophils band forms 4 per cent, segmented forms 64 per cent, total neutrophils 68 per cent, lymphocytes 16 per cent, monocytes 16 per cent

Aspiration biopsy of the sternal marrow revealed no abnormality when performed soon after admission to hospital The urine showed no abnormality Repeated blood cultures were sterile The Widal, Weil Felix and Brucella agglutination reactions were negative The Mantoux reaction, using a dilution of 1/100 old tuberculin, was negative No evidence of any pulmonary lesion was demonstrable radiographically

### *Autopsy findings*

A post mortem examination was conducted 13 hours after death The body was that of a very pale and wasted young woman There were many petechiæ and ecchymoses over the body, particularly on the anterior abdominal wall, the chest, lips, neck and limbs Hypostatic staining was not marked and rigor mortis was present

In the mediastinum were some small reddish lymph nodes The other mediastinal contents appeared normal Each pleural cavity contained 6 oz of blood stained fluid There were diffuse ecchymoses beneath the visceral pleuræ, and apart from slight basal congestion the lungs showed no abnormality The pericardial cavity contained 2 oz of slightly blood stained fluid There were petechiæ in the epicardium and endocardium but no other abnormality was seen in the heart

The spleen weighed 611 g and the consistency was softer than normal No malpighian bodies were discernible to the naked eye The liver was enlarged

(1559 g.) and presented an unusual appearance, being pale and fatty with a bluish discolouration around the larger vessels. The various abdominal viscera all displayed this bluish discolouration but there was no other gross abnormality. The *para-aortic and mesenteric lymph nodes* were *all slightly* enlarged and red in colour.

The sternal bone marrow presented a normal appearance to the naked eye. The femoral marrow was not examined owing to certain restrictions imposed by the relatives.

## *Histology*

*Spleen.* The most prominent feature is the absence of malpighian bodies and lymphoblastic tissue. Hæmorrhages are present throughout. In the medullary portion of the organ there is proliferation of large histiocytes and their precursors. Many of these have irregular large nuclei; some are multinucleate and a few bear a superficial resemblance to Dorothy Reed cells. The sinuses contain these large plump histiocytes which overshadow the ordinary sinus lining cells. Some histiocytes contain a few small droplets of fat while others contain varying numbers of ingested red blood cells. Hæmosiderin is present in the sinus lining cells and to a lesser extent in the large histiocytes lying in the sinuses. In general the architecture of the spleen is preserved but the sinuses are crushed by the great numbers of medullary histiocytes.

*Lymph nodes.* The microscopic picture of the lymph nodes is particularly striking. Follicles have disappeared and in both the follicular and medullary portions there are a few lymphocytes and their precursors. The sinuses are enlarged and the sinus lining cells contain much hæmosiderin. Within the sinuses, too, are free erythro-phagocytic histiocytes. These contain very little pigment.

*Sternal bone marrow.* Ordinary hæmopoietic tissue is very scanty. Throughout are increased numbers of erythro-phagocytic histiocytes.

*Liver.* Extreme fatty change is present. The Kupffer cells are prominent and contain hæmosiderin. Some, in addition contain red blood cells. There are occasional rounded erythro-phagocytic cells in the sinuses which contain no pigment. The fat of the porta hepatis contains erythro-phagocytic histiocytes; these are rather sparse.

*Heart.* There are sparse *erythro-phagocytic histiocytes in the epicardial* fat and in the connective tissue trabeculæ of the myocardium. Adipose tissue from the peri-renal area and in the lumbar vertebral areas contain sparse erythro-phagocytic histiocytes which here and there are aggregated in denser groups.

No sections of skin were examined, and the other organs showed no abnormality.

## *Discussion*

The histological picture is that of a medullary reticulosis in which there is proliferation of erythro-phagocytic histiocytes in the spleen. Elsewhere, erythro-phagocytic histiocytes can be found, but their precursors are not apparent.

This condition corresponds to 4 cases described by Scott and Robb-Smith and to six others they collected from the literature. The features of their cases as in the present case were splenomegaly with slight lymphadenopathy, progressive anæmia and wasting, pyrexia, *terminal purpura and jaundice.* Although the histiocytic proliferation was often confined to several organs, the overall findings were that the disorder was one of the whole lympho-reticular system. They observed that the bone marrow may show a maturation defect in addition to the other changes. In the present case, however, there was a lack of hæmopoiesis in that portion which was examined. This may be an irradiation effect.

According to the concept of hypersplenism as formulated by Doan and Wright (1946) and amplified by Dameshek and Bloom (1948) and Dameshek and Estren (1948), certain normal functions of the spleen become abnormally active thus leading to certain types of anæmia with or without associated reduction in the other blood cells and/or the platelets Two of these functions are inhibition of maturation of the hæmopoietic tissues and hæmolytic activity. According to this concept there are certain diseases associated with hyper activity of the spleen This "secondary" hypersplenism is to be found in certain infections such as tuberculosis, syphilis and malaria amongst others, and certain other disorders such as Gaucher's disease In addition there is a "primary" hypersplenism in which the depressed marrow activity and increased hæmolytic activity lead to panhæmocytopænia Dameshek and Estren stress the functional nature of the hypothesis and state that hypersplenism has no histopathological basis They never observed a generalised erythrophagocytosis.

On clinical grounds, the present case falls into the category of primary hypersplenism in which there is a recognisable anatomical basis

## *Summary*

A case of histiocytic medullary reticulosis has been described The characteristic features of this disorder are progressive cachexia and anæmia, fever, splenomegaly, generalised lymphadenopathy with terminal purpura and jaundice The characteristic anatomical change is erythro phagocytosis and a proliferation of erythro phagocytic histiocytes in the spleen

Thanks are due to the Medical Artistry Department of the University of Sydney for the photomicrographs

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# OBITUARY NOTICES OF DECEASED MEMBERS

## Stephen Roodhouse Gloyne

24th December 1882—25th September 1950

(PLATE LIII)

STEPHEN ROODHOUSE GLOYNE was born in London in 1882. His parents soon returned to their native Yorkshire and he went to school in Wakefield and afterwards to Leeds, where he graduated M.B., Ch.B. in 1906 and M.D. in 1909, being one of the first graduates of the new University. He spent some time at the Middlesex Hospital, first as a student and later in the pathological department, and he also worked in the pathological laboratory at St George's Hospital for the D.P.H., which he took in 1908. After the usual hospital resident posts and a spell of general practice in Selby, he was for a time a school medical officer in Surrey. He rarely went north after this period, though he always regarded himself as a Yorkshireman and he was a faithful supporter of the county side at cricket.

In 1911 he was appointed pathologist to the hospital in Victoria Park now known as the London Chest Hospital and he remained there until his retirement in 1948. He loved that hospital, which he saw through two wars, and his personality is woven into its fabric. His department was at first only a single small room, not even supplied with electricity, but Gloyne had already built up a fine service long before the accommodation caught up with him in 1926.

At the outbreak of the second world war he had a fully equipped building with a large animal house and the Barnes Research Department, which he directed. The museum was already famous for a number of " congenital " hearts collected by Peacock, and Gloyne proceeded to add carefully selected specimens, chiefly of lungs, from his own practice. It was a sad morning for him when, in 1941, he found it in ruins ; only 18 out of 400 specimens were left and he remarked that his life-work had gone up in smoke. Gloyne quickly won for himself affection and respect from the honorary staff, and his little laboratory with its large open fire became a meeting place for consultants and residents alike, a habit which was not broken in the subsequent removal to larger premises. Later he became Dean of the Postgraduate Association and he took great pains in organising the teaching at the hospital.

When he retired he was given a grant by the Medical Research Council to work on the pathology of industrial pulmonary disease, and, since this study was based on the statistical analysis of post-mortem records, he elected to work at the Institute of Social Medicine. The grant was a double joy to him. He felt that there was still a niche for him in the medical world, and he could be in Oxford. Once, when browsing round the Bodleian library with him, one caught the impression that he was sensible of having missed something in his early life. Oxford would have meant a great deal to Gloyne and it was a happy chance that sent him there in the end. At the time of his death he was also acting as consulting pathologist to the King Edward VII Sanatorium, Midhurst.

In 1919 the journal *Tubercle* was founded and for the first year the editorial board was anonymous. In the second volume Gloyne's name appears as editor and he continued to occupy this office until 1937. The journal was originally intended for everyone interested in tuberculosis, whether lay or medical. Gloyne himself attached considerable value to the bibliography, which, month by month, kept readers in touch with the world literature of the subject. Gradually *Tubercle* came to cater more and more for medical readers and in 1946, with Gloyne still an advisory member of the board, it became the organ of the British Tuberculosis Association, a purely medical society.

Gloyne, however, never lost interest in the problem as a whole and his book, *Social aspects of tuberculosis*, was based on lectures which he gave for many years at the Royal College of Nursing. He had a respect for the work of nurses and almoners which was all part of his concern for the welfare of the patient. His pathological papers show orthodox scientific detachment, but his choice of subjects reveals a bias in favour of a fairly direct human contact, and in 1948 he was given the Weber-Parkes prize which is awarded by the Royal College of Physicians for work in the field of tuberculosis.

Gloyne's name is usually linked with tuberculosis and the pneumoconioses, especially asbestosis. Interest in the latter was kindled by the proximity of a factory which kept him well supplied with material. He made good use of the opportunity and published, in easily accessible journals like *The Lancet* and *Tubercle*, excellent descriptions of the morbid anatomy of naturally acquired asbestosis. The large number of cases involved drew attention to the hazard and was probably in some measure responsible for the improved safeguards which have been put into operation. He also did some experimental work on the subject and was the first to demonstrate the fine fibre of asbestos running through the centre of the asbestos body. One of the problems he discussed was the exact significance of asbestos bodies in the sputum, as he did not like hasty verdicts of disability to be based solely on their presence, which he regarded as a sign of tissue reaction and a danger signal, but not necessarily proof of progressive fibrosis. It was an important distinction for employers and gave hopes of recovery if such workers were removed from the industry.

Gloyne was always on the look-out for the clinical implications of his observations and he often talked about combined diseases which he had noticed in his routine work. The association of tuberculosis with asbestosis, syphilis, heart disease, anæmia and vitamin deficiency was the subject of various short papers. He was an observer, little prone to theorising, and did not permit himself to draw very definite conclusions from these associations, so he will not have much to blush for when the Recording Angel confronts him with his written word.

He did have strong opinions about some things, and milk was one of them. For many years he examined milk for the Essex County Council and as usual he illuminated a routine job by careful scrutiny of his results and by experimenting, principally with the tubercle bacillus. He made it his business to find out details of the dairy industry and from this intimate knowledge he developed positive but not always printable opinions. Quite briefly his researches led him to the conclusion that tuberculosis-free cattle were supremely important to the farmer. They were also desirable for the human population, but pasteurisation was and always would be necessary in any case. Gloyne was very anxious that infections of milk other than tuberculous should not be neglected by the profession. His experience was that there was a real danger of *Br. abortus* and streptococcal infection of man, even from the best herds. For his own grandchildren he insisted on the provision of milk which had been well and truly boiled.

Gloyne knew a great deal about the histology of the lung in health and disease and was recognised as an authority on pulmonary pathology. He was a member of the Medical Board for Silicosis from 1931 till 1948 and he continued to examine sections for the Board after his retirement. In 1938 he wrote the chapter on pathology for the book *Silicosis and asbestosis* edited by Lanza. His little monograph on thoracic puncture fluids written in 1930, though not pretending to be comprehensive, is still one of the best accounts in English.

It sometimes happens that a pathologist deliberately narrows his field so that he comes to be on closer terms with clinicians within that field than with those doing general pathological work. Gloyne was intimate with physicians and surgeons concerned with thoracic disease. He had been President of the British Tuberculosis Association and a Vice-President (by his own wish) of the Thoracic Society, but he was not personally known to many pathologists and it is on his work that he would wish to be judged by them. Gloyne really lived for his work: all day and half the night was his habit. He had a journalistic streak and was a regular writer of leaders and annotations. " Something for *The Lancet* " was always on hand, requiring a basinful of midnight oil for its completion. He had a talent for extracting the essence of other men's papers and adding observations of his own which made his articles both interesting and easy to read.

He had no recreations other than exploring Cistercian abbeys and medical history, and it was a great satisfaction to him when his life of John Hunter was in the press. The book is a delightfully discursive account of Hunter and his environment and only a man with a very retentive memory could have called to the service of his pen so many literary and historical allusions. It is more than a portrait, it is a conversation piece, cleverly composed to give a balanced picture of Hunter's place in medicine, and Gloyne's choice of extracts from the letters to Jenner light up the nature of the man in a few lines.

Gloyne was a very kind man. He had a friendly habit of introducing people to each other at meetings which was most helpful to newcomers, and his toleration of human frailty came in useful in the hospital community. He could stand fools gently if not altogether gladly and in his unobtrusive way he did a great deal for the understanding of clinically applied pathology. One feature of his publications was his acknowledgment at the end of the help of his chief technician, and Mr Smart's name always appeared in the annual reports of his department. It was very dear to his heart that the skill and loyalty of technicians should be properly recognised when pathologists take to print. He was also a zealous man. Finding himself at an E.M.S. hospital near London with some students at a loose end, he immediately set about organising classes and soon had teaching in progress, roping in neighbouring Army and Air Force personnel, either for giving or receiving instruction.

Gloyne and his wife joined the Society of Friends shortly after they married and he was buried at Jordans. Many Friends testified to his unfailing benevolence to anyone in need of help or encouragement.

It is not given to many to be major prophets but lesser men also may have an abiding influence for good and Gloyne had the quality that endures.

For some years he had suffered from diverticulitis and he had recently had a coronary thrombosis. But he could not bring himself to give up working, and in the end had a cerebral hæmorrhage. He is survived by Mrs Gloyne and a son and daughter.

Norah H. Schuster

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(1931) which he wrote in collaboration with Dr Max Cutler, the substance of his observations and conclusions will be found. Recognition of the value of his contribution in the campaign against cancer was the award by the Royal College of Surgeons in 1931 of the Walker Prize, and later he was honoured by the United States of America by an invitation to carry out an extensive lecture tour in the principal citadels.

As a surgeon, as a teacher of the undergraduate and as a supporter of the academic life of the student he was equally zealous. Whatever Cheatle undertook he pursued with thoroughness. He could rise to any occasion on which his services might be most needed, as his records in the South African and 1914-18 Wars bear testimony; in the first he was Consulting Surgeon to the forces in the Field and was awarded a C.B.; in the second he was Consulting Surgeon to the Royal Navy, and appointed Surgeon Rear-Admiral. The standard he set himself was the highest and he encouraged all his co-workers in the pursuit of ideals as high as his own. JOHN EVERIDGE

# BOOKS RECEIVED

## Virus and rickettsial diseases

By S P BEDSON, A W DOWNIE, F O MACCALLUM and C H STUART HARRIS 1950 London Edward Arnold & Co Pp viii and 383, 35 text figs 24s

In spite of the outstanding importance of some of the virus infections of man, many clinicians and medical students still look on virology as an esoteric science of relatively little interest to the practising physician The blame for this unfortunate attitude, as the authors of the volume admit in their preface, must be attributed chiefly to the research worker for failing to present the results of his work in acceptable form The four authors, each of whom is a distinguished researcher in the virus field, deserve great credit for rectifying this position, they present lucid and accurate accounts of practically all the known human virus diseases in a form which should both arouse and maintain the interest of the reader

To understand the wide range of dissimilar phenomena and the difficulties of applying measures of control in the virus diseases, some knowledge of the general characteristics, ecology and immunological reactions of the viruses themselves is necessary The first four chapters provide this essential background and, by frequent comparisons, clarify the similarities and dissimilarities between virus and bacterial behaviour Chapter five gives a good account of the rickettsiæ and the typhus group of fevers The various virus diseases are then dealt with individually Those in which the ætiological agents have not yet been isolated necessarily receive cursory treatment, but the others are adequately discussed from the aspects of clinical and pathological picture, virus characteristics, laboratory diagnosis, epidemiology, control and treatment The last chapter is devoted to a general consideration of the bacteriophages and is included chiefly because of the great contributions which bacteriophage studies have made to our understanding of host virus relationships The practical importance of the phages in epidemiological investigations has, moreover, not been overlooked and is well illustrated by references to the phage typing of *Bact typhosum* and of staphylococci As an appendix the available diagnostic tests and the materials from patients required for their performance are summarised in tabular form

The book should prove a useful addition to the library of the practising physician It will also be invaluable to the medical student who hitherto has had no ready means of acquiring a balanced view of the place of virology in medical science and practice For the keen student references appended to each chapter offer access to more detailed information The authors have obviously had no intention of catering for virus research workers, but even these will not fail to obtain an adequate return for the modest outlay required to secure the volume

### The pathogenesis and pathology of viral diseases

Edited by John G. Kidd. 1950. New York; Columbia University Press: London; Geoffrey Cumberlege. Pp. xx and 235; 107 text figs. 32s. 6d.

This volume contains fourteen contributions to a symposium held at the New York Academy of Medicine in December 1949. The title has been widely interpreted for the papers deal with such varied subjects as:—the spread of viruses from infected to susceptible hosts (Shope), culture and effects of viruses in chick embryo cells (Buddingh), relationship of viruses and cells with particular reference to the interference phenomenon (Dalldorf), electron microscopy of viruses (Wyckoff), activators of viruses by absorption co-factors (Anderson), proliferative lesions caused by viruses and virus-like agents (Kidd), and the nature and pathogenesis of neuronal changes in poliomyelitis (Howe). Most of the articles contain much original material and the reviewer was particularly impressed by the discussions on cardiac lesions produced by viruses (J. M. Pearce) and on the pathogenesis of poliomyelitis. The quality of many of the photomicrographic illustrations is particularly good. Much of the material here collected is not to be found elsewhere and this little volume should be a source of interest and pleasure to every worker in the field of virus diseases.

### Introduction to the bacteria

By C. E. Clifton. 1950. New York, Toronto, London: McGraw-Hill Book Company, Inc. Pp. xii and 528; 180 text figs. 42s. 6d.

Until fairly recently the study of micro-organisms has tended to be conducted in a number of separate compartments and, in Great Britain at least, it has been heavily weighted in favour of medical bacteriology. Furthermore, biochemical and biophysical studies have lagged behind description and classification, and these again behind the more utilitarian study of practical application and diagnosis. The author of this useful and timely *Introduction to the bacteria* writes in his preface:—" Bacteriology has progressed from a primarily descriptive science to one in which serious attempts are being made to interpret the bacteria and their behavior on the basis of modern biological, physical and chemical concepts. In particular, in recent years the application of biochemical principles to the study of the bacteria has considerably enriched our understanding of these organisms". The book " is the result of endeavors over many years to digest and to condense for the general student the concepts of the what, the how and the why of bacteria and bacterial activity in as simple and brief a presentation as appears to be consistent with the development of a fundamental understanding of the bacteria and related forms of life". The author ranges beyond the bacteria proper and includes chapters on protozoa, algæ and fungi. In a sense, therefore, the book is an introduction to the study of microbiology in general with the main emphasis on bacteria.

After an introductory chapter, which is partly historical, there are largely descriptive chapters on the observation of bacteria (microscopy, staining properties, differential stains), structure (including modern work with the techniques used by Robinow, Bisset and others and with the electron microscope), protozoa and algæ, fungi, bacteria, with descriptions of the orders of the class Schizomycetes, and viruses. Then follow chapters dealing mainly with biochemistry and biophysics, *e.g.* energy requirements of bacteria, mechanisms of microbial respiration, metabolic groups and multiplication and death of bacteria These chapters demand a relatively advanced knowledge of organic chemistry Next comes a chapter on microbial variation and another on the classification of the Eubacteriineæ and the use of the Bergey system Whatever doubts some bacteriologists may have about the classifications chosen in certain parts of *Bergey's Manual*, its all embracing character justifies its use when bacteria outside the medical field are considered, as they must be in a book dealing with general bacteriology Chapters on the bacteriology of soil, water and air, on food preparation and preservation and on industrial microbiology (fermentations by bacteria and moulds) provide a survey of many fields of applied microbiology The last four chapters cover infection and resistance, serological reactions, Enterobacteriaceæ and microbiology of infectious disease The chapter on the Enterobacteriaceæ serves as an example of the methods used in isolating and identifying members of a large and complex group of organisms which includes both pathogenic and non pathogenic species It demonstrates many principles of bacteriology which can also be applied to other families

The numerous topics covered in order to give a comprehensive picture of modern bacteriology can clearly be treated only at an elementary level within the 528 pages of this book Some chapters are weaker than others, for example those on infection and resistance and on serological reactions which give 38 pages in all to these wide subjects But this may be enough for the elementary student Perhaps the most valuable achievement of the book is to have united classical, descriptive bacteriology with modern microbiological chemistry for the student at the start of his studies Special topics can later be studied more intensively from specialist books It is the beginner who has not hitherto been catered for enough by the provision of an all round view of general microbiology

The book is on the whole well illustrated, although some of the photographs could be more informative and some omitted without loss The picture of Erwin F Smith on p 510 bears a striking resemblance to Theobald Smith A rather large number of spelling mistakes has escaped the proof reader and there are some errors of fact These imperfections will, it is hoped, be removed at the reprinting or in the new edition which will doubtless be demanded, since it is clear that this book will prove very useful

## The diagnosis of Salmonella types

By F Kauffmann 1950 Springfield, Ill , Charles C Thomas Oxford, Blackwell Scientific Publications, Ltd Pp vii and 86 16s 6d

This book describes the practical classification of Salmonella by serological and biochemical methods The methods are those used by the author at the International Salmonella Centre at Copenhagen and are for the most part standard practice, but the author's view of slide agglutination as " a technique (which) gives such definite results that in most cases test tube agglutination is superfluous " would not be accepted by most British bacteriologists In the reviewer's opinion the slide technique, provided suitably absorbed sera are used, is adequate for routine diagnostic work But the tube test is essential for absolute identification and for the identification of new types, where semi quantitative as well as qualitative tests are required In view of the reliance the author places on the slide test it is strange that practically no reference is made to the occurrence of normal antibodies in rabbits because they conceal and obscure the basic fact that the phenomena of anaphylaxis are the result of the reaction of antigen with antibody and misleading because they have given rise to theories which lack support other than that derived from a fantastic nomenclature. The technique of anaphylaxis is described under three headings, the preliminary sensitisation, the incubation or pre-anaphylactic period and the second or shocking dose of the antigen. The literature of passive anaphylaxis and the influence of the recorded results on the theories of anaphylaxis are admirably reviewed. The Arthus' phenomenon is discussed. There follow valuable chapters on recorded observations of anaphylaxis in man, in mammals, in birds and in vertebrates. The difficulties which have resulted from observations recorded under the misleading title of Anti-Anaphylaxis are discussed. There are chapters on desensitisation and on the suppression of anaphylactic manifestations by anæsthetic and narcotic drugs, by drugs which suppress the action of histamine and by the prophylactic injection of normal serum.

This is an excellent book which comprises within 187 pages a concise and critical survey of the more important papers on anaphylaxis which have been published during the last fifty years. It begins at the beginning of this difficult subject and gives a clear, critical and readable account of the steps by which our knowledge of anaphylaxis has been obtained. It provides within a small volume an introduction to anaphylaxis for the reader who is unfamiliar with the subject, and a concise work of reference which will be valued by the experienced serologist.

## Lehrbuch der gerichtlichen Medizin

By ALBERT PONSOLD. 1950. Stuttgart: Georg Thieme. Pp. xx and 568; 169 text figs. DM 49, $11.65.

The editor of this book, Professor A. Ponsold, is also the largest single contributor. Altogether, there are seventeen contributors and this co-operative effort shows both the advantages and disadvantages of this method of book production. The individual chapters, some of them highly specialised, are the work of experts in the respective fields and can therefore be taken as authoritative; but there is considerable inequality of treatment such as can scarcely be avoided where so many experts are concerned. For example, some sections are fairly well documented with references to original papers; others give a few key references; others give none at all.

The book is addressed to both the medical and the legal professions, the legal side having, naturally, particular reference to German law. Whilst this limits the value of the book for the non-German student, it is of value to those with an international practice necessitating a knowledge of German medico-legal relations. Moreover, it is of more general interest because of the discussions in the first two chapters—one on medical ethics which includes a section on the difficult problem of euthanasia, and one on the medico-legal aspects of psychiatry.

In general, the book follows along conventional lines, which makes it of no particular value to the non-German medical student whose purposes are better served by various books in his own language and related to his own system of laws. It may, however, interest teachers of the subject, since it provides certain novelties in presentation. Thus whilst conventional photographs of post-mortem appearances, both naked eye and microscopic, are included amongst the illustrations, there are many clear line drawings and purely diagrammatic sketches of the type with which American text-books have familiarised us, and while some of them seem unnecessary, most are excellent for the purpose of presenting useful facts in a striking way.

The discussion of the blood groups including the Rh factor is quite well done and as clear as the present state of the subject permits. It is comprehensive and takes account of British and American work with a good selection of key references.

The chapter on hairs which was written by Lochte contains no illustrations, and there is a complete absence of any reference to the important subject of toxicology.

Taking it all round, the book is well printed and presented and well illustrated in general.

### Steroid hormones and tumors

By Alexander Lipschutz. 1950. Baltimore; The Williams and Wilkins Company: London; Baillière, Tindall and Cox. Pp. xiv and 309; 234 text figs. 46s. 6d.

The foundation of this book is a collective investigation by Dr Lipschutz and several colleagues into whether, where and how the steroid hormones may interfere in the dynamics of cancer. For the practical work in this research the guinea-pig was employed. In this animal a characteristic effect of œstrogen is the induction of fibroids of the peritoneum; and these tumours have been used by Dr Lipschutz as criteria in numerous quantitative experiments. The highly important results of these inquiries have been co-ordinated with the general body of our knowledge in the field of cancer, and this has been done with great care.

Perhaps the most valuable discovery made in Dr Lipschutz's laboratory has been that to cause peritoneal fibroids in the guinea-pig an unremitting supply of œstrogen is essential, and not as might have been supposed excessive quantities provided at separate intervals of time. Remarkably small amounts of œstrogen will cause these tumours provided that it is available without cessation, and to ensure a constant supply the drugs to be tested were used in the form of pellets. Most workers in this field will support Dr Lipschutz in extending this ætiological detail to the occurrence of tumours in the breast, uterus and other organs. Another noteworthy feature of this book is the emphasis which has been given to hormonal imbalance as an important and perhaps essential factor in the causation of tumours by steroid hormones. Though primarily concerned with experimental work on guinea-pigs, the varying forms of neoplasia in other species are not forgotten, and in no way can the work or conclusions of Dr Lipschutz and his colleagues be neglected by clinicians. The book is a store of information which is made readily available by a comprehensive bibliography and a good index.

### Micro-analysis in medical biochemistry

By E. J. King. 2nd ed., 1951. London: J. & A. Churchill Ltd. Pp. viii and 222; 3 figs. on 2 plates and 19 text figs. 14s.

This edition is very similar to the earlier one, but additions have been made to bring it up to date, and consequently the size and price are somewhat increased.

Undoubtedly the best part of the book is that which deals strictly with micro-analysis, a field in which the work of the author and his colleagues is well known. But the book includes more than the title suggests and this is not so well done. The analysis of calculi, for example, occupies little more than two pages and refers only to biliary and renal calculi. Carbonate is referred to as a frequent constituent of the latter, but in man its presence in any appreciable quantity is rare; triple phosphate, which is a common constituent, is not mentioned. The description of the tests for proteins in urine is incomplete. Bence-Jones protein does not always behave in the manner referred to, and no detailed instructions for its detection are given. It ought, moreover, to precipitate at 40°-60° C., and not at 60°-70° C.

Where the book fails is in regard to the clinical interpretation of laboratory results. Abnormalities in the composition of human blood are given in a table occupying less than one page; pathological changes in the cerebrospinal fluid are indicated in a table which takes up only one-third of a page. Some references to clinical applications are also made before describing methods of analysis, but in general these add little to what is in the tables and what they add is sometimes of doubtful value, displaying a lack of balanced judgment. Certain important non-renal causes of raised blood urea receive no mention, but the increased excretion of amino-acids in the urine in the Fanconi syndrome is included. The only cause given for low sodium concentration in the blood is Addison's disease. Under plasma proteins nothing is said of the way in which these may be affected in liver disease, except in the table, where hepatitis is included as one of three causes of raised plasma globulin and myelomatosis is not. The statement is made that higher values than normal for urinary diastase are found in "diseases of the pancreas", no distinction being made, for example, between acute and chronic pancreatitis. The author admits in his preface that what is said in this way "may appear cryptic and inadequate to the clinician" but justifies himself on the ground that laboratory workers, clinical pathologists and students have found these "brief reminders" useful in recalling what they have previously learnt but in part forgotten. But surely this can only be true if what they have forgotten is just those few things that the author has decided to remind them of.

The book is really intended as a practical handbook for laboratory workers and it serves this purpose well so far as micro-analysis is concerned.

### Microdiffusion analysis and volumetric error

By Edward J. Conway. 3rd ed., 1950. London: Crosby Lockwood & Son, Ltd. Pp. xxiv and 391; 75 text figs. 25s.

The second edition, published in 1947, has already been reviewed in this *Journal* (vol. lix, p. 514). New matter appearing in the present edition of particular interest to pathologists includes methods for the determination of alcohol in blood and urine, of carbon monoxide in blood and of chloroform in blood. All these use the well-known Conway microdiffusion technique and apparatus. The determination of ammonia, of total nitrogen, of urea and of carbon dioxide receive very full treatment in this edition. The theoretical aspects of microdiffusion analysis are of necessity treated mathematically, and will present difficulties to some readers, but this need not deter them from taking advantage of the practical part, for actual methods are clearly and simply described. The last part of the book, on volumetric error, is of considerable importance but, for many readers, of considerable difficulty also Many workers who have to carry out volumetric analyses would be grateful for a simpler and less mathematical treatment of the subject, or alternatively, a mainly non-mathematical summary of the principles involved and the conclusions reached.

**Bibliography of electron microscopy**

Edited by V. E. COSSLETT for the Institute of Physics. 1950. London Edward Arnold & Co Pp 350. 40s.

Electron microscopy is now applied to most branches of science and has already acquired an extensive literature The Electron Microscopy Group of The Institute of Physics has rendered a great service by classifying most of the papers published up to the end of 1948. By reading the present book, the research worker is able to assess what new advances in his own field have come from this powerful aid to fine structure analysis The biological sciences are well represented and many papers are listed, dealing with bacteria, viruses, proteins, tissue structure, etc , and the bibliography should prove valuable to medical research workers who collaborate with the electron microscopist Perhaps one fault is that indexing is by author's name rather than by subject matter, but this is no doubt unavoidable, for if a given paper interests research workers in more than one branch of science, much cross reference would otherwise be necessary.

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# SUPRARENAL HÆMORRHAGE AND NECROSIS IN PREGNANCY

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(Plates LIV and LV)

In recent years there have been numerous reports of single cases of suprarenal hæmorrhage or "apoplexy" associated with pregnancy and the puerperium (Hall and Hemken, 1936; Keele and Keele, 1942; Dodds, 1945; Hurter, 1945-46; Burnett, 1948; Arnold, Richer and Lepore, 1949). Other workers have noted hæmorrhage and necrosis in the suprarenal cortex in association with fatal complications of pregnancy, *e.g.* with severe eclampsia (Way, 1947), with accidental hæmorrhage (Chirié, 1908) and with renal cortical necrosis (Jardine and Teacher, 1910-11; Evans and Gilbert, 1936; Reyna, 1936; Hertig, 1946). It therefore seems worth while to present a study of the post-mortem material from fourteen pregnant or puerperal cases in which suprarenal hæmorrhage with or without necrosis of the cortex was found. Twelve of the cases were seen during ten years (1936-1946) at the Glasgow Royal Maternity Hospital and two were seen more recently. The suprarenal lesions varied widely in type and severity from case to case. Only cases in which these lesions were a striking feature have been included and an attempt has been made to group them according to type of lesion and possible cause. Brief accounts of the clinical and pathological features of each case are given in an appendix. In none of the cases was the suprarenal lesion diagnosed during life and therefore much relevant clinical investigation is lacking.

## Pathological findings

The condition was easily recognisable at autopsy because of the hæmorrhagic appearance of the suprarenals on surface and section.

In severe early cases the glands were swollen and plum-coloured, but in the later stages they tended to be more brick red. There was often an associated retroperitoneal œdema which was sometimes blood-stained and sometimes spreading down over the front of the kidneys. In over half of the cases both suprarenals were equally involved. In the remainder one gland was very much more affected than the other but a completely unilateral lesion was found in only one case. The lesions were always confined to the suprarenal cortex; the medulla was sometimes congested but never showed significant pathological changes.

On microscopic examination the lesions in any particular case appear to be all of about the same duration, but there are obvious differences between the duration of the lesions in different cases. From a study of these fourteen cases it has seemed possible to discern two main histological types of lesion. These are described here according to their apparent duration as judged histologically.

1. *Multiple hæmorrhagic lesions.* These lesions vary greatly in severity from case to case and even from one gland to another in the same case; they range from small patches of hæmorrhage and extreme congestion of the zona fasciculata (fig. 1) to complete disruption of the gland by hæmorrhage—a true "apoplexy" (fig. 2).

In the milder cases (cases 1, 3 and 4) the initial lesion can be studied more clearly. The hæmorrhages often appear related to masses of enormously swollen, vesicular, cortical cells, some of which show signs of disintegrating (fig. 1). In severer cases (cases 2, 6, 8 and 14)—mainly those after delivery or fits—the hæmorrhages are larger and confluent so that the entire cortex may be involved and the columnar structure broken up.

In patients that survive the lesion for 24 hours (cases 2 and 8) the cortical cells show signs of degeneration near the centre of the lesion. Their nuclei become shrunken and dark and begin to fade and the red corpuscles in the central parts of the lesion lose their hæmoglobin although those at the periphery appear normal. Deposition of a yellow crystalline pigment can be seen around the lesions and some polymorphonuclear infiltration occurs at the periphery. At a still later stage—about 36 hours—the necrosis of the cortical cells becomes the outstanding feature (case 6). Some of the nuclei of the cortical cells in the lesions show smudgy pyknosis, while others stain very poorly or have disappeared. There are only a few red cells left in the area and those that are present are devoid of hæmoglobin and appear to be degenerating. The necrotic area is usually surrounded by a zone of congestion and hæmorrhage; the cells of the reticular layer appear to be healthy although this zone may be so grossly congested that individual cells appear to be lying in blood spaces.

2. *Necrotic lesions.* This group consists partly of cases which survived the initial lesion longer than those of group 1, the apparent

SUPRARENAL HÆMORRHAGE AND NECROSIS

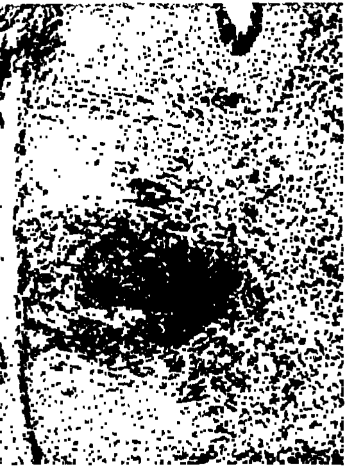

FIG. 1.—Case 4. Suprarenal cortex showing small hæmorrhage adjacent to clear celled areas. Hæmatoxylin and eosin. ×25.

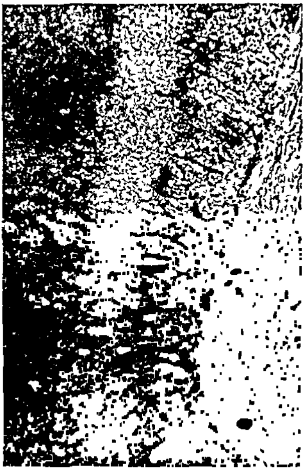

FIG. 3.—Case 5. Suprarenal cortex showing a band of necrosis with congested edges. H. and E. ×45.

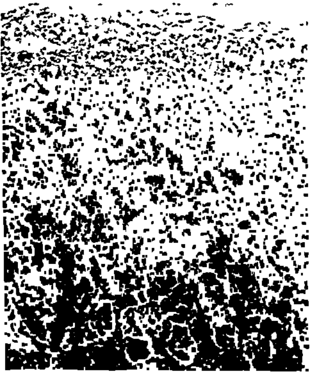

FIG. 2.—Case 6. Suprarenal cortex showing disruption by hæmorrhage. H. and E. ×100.

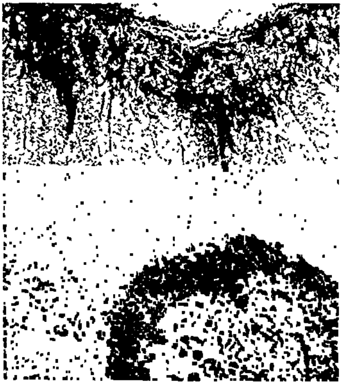

FIG. 4.—Case 11. Suprarenal cortex showing massive necrosis with loss of hæmoglobin in mid-cortex. Surviving medullary cells are present. H. and E. ×50.

duration varying from about 36 hours to 9 days. The suprarenals show massive necrosis of the cortex. In cases 5, 9, 10 and 13 the lesions are patchy and surrounded by a congested zone (fig. 3) while in the remaining cases (cases 7, 11 and 12) the whole cortex appears to be necrosed except for a few groups of cells in the zona glomerulosa (fig. 4). In the lesions of longest duration (case 7, nine days ; case 12, seven days) nuclear staining is completely absent from the necrosed area except for a zone of karyorrhectic nuclear dust beneath the zona glomerulosa : the cortical cells remain as ghosts within the columnar fibrous structure which is not disrupted. In case 7 there is considerable endothelial and fibroblastic proliferation in the surrounding tissues ; no remains of hæmorrhage can be seen and there is no polymorph infiltration. In the less advanced cases (cases 5, 9, 10, 11 and 13) with lesions estimated as from 36 hours to 6 days old, the nuclei of the cortical cells in the affected areas are seen in varying stages of disintegration. Considerable polymorph infiltration is seen in cases 9, 10 and 11 but not in the others. Only in the cases of about 36-48 hours' duration (cases 5, 9, 10 and 11) can red cells still be seen in the necrosed areas and they all show loss of hæmoglobin (fig. 5).

Fibrinous thickening of the sinusoid walls in the congested areas surrounding the lesions was noted in a few cases in each group and red granular thrombi were present in some of the sinusoids in the necrosed area in the case of longest duration (case 7, nine days). These were of a secondary change in the development of the infarct. Arterial thrombi were not found in any case.

Venous thrombosis was found in only three cases of the present series (cases 6, 8 and 12). In case 6 there was recent thrombosis of the central vein of the left gland and in this gland the cortex was disrupted and contained massive hæmorrhages. There were numerous hæmorrhages in the right gland but its vein was empty. In case 8 there was white thrombus in both suprarenal veins and both cortices were grossly congested and showed some absorption of the cortical cells. In case 12 there was thrombosis of the left suprarenal vein, while the left gland showed only slight hæmorrhagic lesions. In the right gland the main central vein was empty but smaller medullary veins were blocked by thrombi and here the entire cortex was in a state of advanced necrosis.

## PATHOGENESIS AND ÆTIOLOGY

No obvious local primary lesion has been found in the present series to account for the suprarenal hæmorrhages and necrosis. By analogy with renal cortical necrosis a vascular catastrophe may be surmised to be its direct cause although arterial lesions were not found. Thrombosis of the central vein or its tributaries has been described in several of the reported cases (Hall and Hemken ; Keele and Keele ; Arnold, Richer and Lepore) and in each the thrombosis is considered to be a primary factor; Hall and Hemken state that venous thrombosis, due to various conditions, is the commonest cause of suprarenal hæmorrhage in adults. This conclusion, however, was reached from studies on single cases and is not supported by the findings in the present fourteen cases. In only three of the fourteen cases of the present series was venous thrombosis found. Furthermore, thrombosis of the central vein has been observed in one obstetric patient, not included in this series, who had no suprarenal lesion at all. It would appear, therefore, that thrombosis of the central suprarenal vein may occasionally be found in association with suprarenal damage but that the two findings are not necessarily interdependent although both may be the result of a common cause.

The condition does not appear to be related to labour, for six of the fourteen patients were undelivered or had suprarenal lesions which quite clearly dated from before delivery. One outstanding predisposing factor would appear to be toxæmia of late pregnancy—particularly vomiting of late pregnancy. In five of the present cases (cases 4, 5, 6, 11 and 12) there was very severe vomiting of late pregnancy with consequent metabolic disturbances, such as dehydration, acidosis, increased blood concentration and azotæmia; cases 5 and 11 also showed severe anæmia which is sometimes associated with late vomiting and in cases 4 and 12 signs of Wernicke's encephalopathy were present. This finding is of special interest as Supplee, Bender, and Kahlenberg (1942) found suprarenal hæmorrhage and necrosis in rats kept on a diet deficient in pantothenic acid; sudden death without signs of impending collapse was frequent among the rats. Eclamptic convulsions occurred in cases 1, 2 and 3 and there were elcamptic liver lesions in cases 4 and 14. Thus in nine of the fourteen cases there was some severe metabolic disturbance.

During the period in which the present cases occurred, patients with hypertensive toxæmia of pregnancy were extremely common in the wards of the Glasgow Royal Maternity Hospital but cases of vomiting of late pregnancy were relatively rare. The number of cases of the latter condition found in this series is therefore striking. The incidence of eclampsia (21 per cent.) is approximately the same as its incidence in the total number of autopsies performed in the Glasgow Royal Maternity Hospital during the same period, and the incidence of suprarenal lesions found in fatal eclampsia is about 2·5 per cent. This is a very different incidence from that found by Way who noted suprarenal lesions in 11 out of 33 cases of fatal eclampsia (33 per cent.).

Another factor of possible ætiological significance is infection. In six of the patients (cases 8, 10, 11, 12, 13 and 14) there was some clinical or post-mortem evidence of infection but unfortunately bacteriological examinations were not made. These cases may, therefore, be related to the well-known Waterhouse-Friderichsen syndrome.

SUPRARENAL HÆMORRHAGE AND NECROSIS

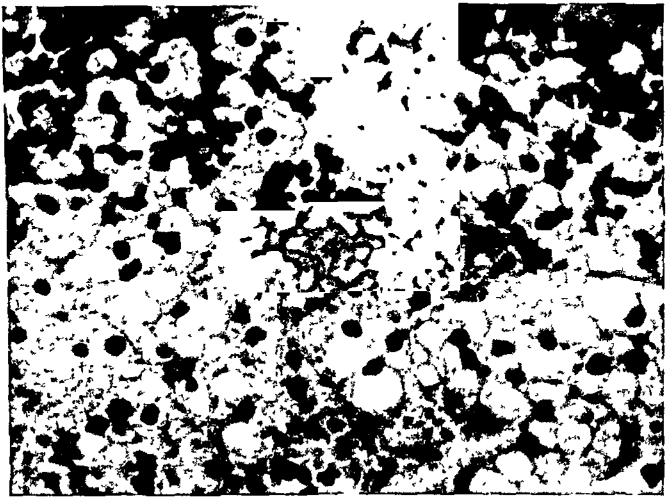

FIG. 5.—Case 11. High magnification of the edge of the hæmoglobin-freed area shown in fig. 4. In the upper part of the figure the red cells stain normally but below they stain faintly. H. and E. ×600.

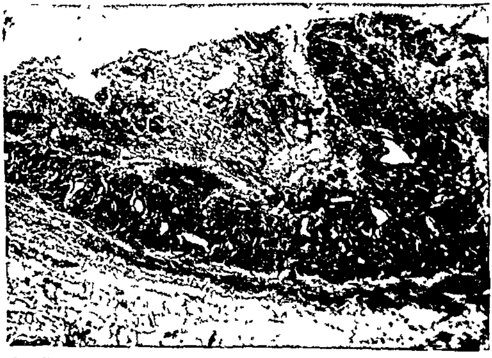

FIG. 6.—Case of Addison's disease. Surviving suprarenal medulla amongst scar tissue lying in perinephric fat. H. and E. ×25.

The final factor which must be noted is that all the patients of this series, with the exception of the three eclamptic cases, were given intravenous infusions of either glucose-saline or blood or both and in some the transfusions were given at a time when, as judged histologically, the suprarenal lesions might have originated. The question of the absolute sterility of these transfusions must be raised. They were given to patients extremely debilitated as a result of vomiting, or to patients who had some clinical evidence of infection and many of whom had suffered severe blood loss: it is therefore possible that grave damage was caused either by a few organisms injected with the fluids or by organisms swept into the general circulation from the uterine veins as a result of circulatory changes.

The condition is in some ways analogous to renal cortical necrosis which can be produced experimentally with staphylococcal toxins and it resembles closely the Waterhouse-Friderichsen syndrome which occurs in meningococcal septicæmia; it is, therefore, possible that organisms or a preformed toxin in the injected fluids might be the cause of the suprarenal cortical damage in some cases.

## CLINICAL COURSE

*Signs and symptoms.* It will be seen from the case notes given in the appendix that the patients suffered from a great variety of the most serious complications of pregnancy and this makes it very difficult to unravel the clinical effects of the suprarenal lesion. Keele and Keele give a detailed description of the right-sided pain in a patient with a right-sided lesion and this, with repeated vomiting and the absence of abdominal rigidity and shock, they consider to be the syndrome of vascular catastrophe in the suprarenals. In the present series, cases 1, 6 and 7 were recorded as complaining of transient severe backache and cases 4 and 8 of transient severe epigastric pain. In these five patients the histological stage of development of the suprarenal lesions at death was such that the initial lesion could well have occurred at about the time of the pain. The absence of any record of pain in other patients may not be significant; backache is such a common complaint of obstetric patients at about the time of delivery that it is not usually noted in clinical histories. Vomiting, sometimes blood-stained, was noted in several cases. In three of the present series and in the case reported by Hurter the symptoms came on suddenly during or immediately after blood transfusion, closely resembling the effect of a mis-matched transfusion.

*Late effects.* Death associated with destructive lesions of the suprarenal cortex in non-obstetric cases may occur in various ways. In experimental animals, bilateral adrenalectomy results in death after a few days during which the animals show a rapid decline to severe asthenia; there is loss of appetite, nausea, diarrhœa, hæmo-concentration and a fall in blood pressure. The Waterhouse-Friderichsen syndrome—fulminating meningococcal septæmia associated with extensive hæmorrhages in the suprarenal cortex—is an illness lasting usually only a few hours and ending in sudden death. Chronic suprarenal insufficiency, associated with long-standing and often gross destructive lesions of the cortex, may be manifest as Addison's disease ending usually as a " crisis ", or it may be symptomless ending in sudden and unexpected death after slight malaise for only a day or two.

The question arises in the present series as to how far death is to be attributed to the suprarenal lesions. None of the patients had total destruction of the cortex of both glands so that it is not surprising that the adrenalectomy syndrome did not occur. None of the cases had survived long enough to develop Addison's disease. Three of the patients (cases 1, 2 and 3) died in coma due to other conditions and the suprarenal lesions do not appear to have played any significant part in the issue. On the other hand, death was due to sudden and unexpected collapse in nine of the fourteen cases reported though certainly some of the patients were already seriously ill. This similarity in the type of death to that in the usual Waterhouse-Friderichsen syndrome and in the non-Addisonian suprarenal destructions suggests that at least the suddenness of the death may be related to the suprarenal lesions. In the remaining two cases death was due to severe and prolonged shock which did not respond to the usual treatment and it is possible that the suprarenal lesions may have prevented recovery. It is, of course, clear that the cause of the suprarenal lesions may have been the actual cause of death in many patients.

It is clear that patients may recover from quite extensive hæmorrhage and necrosis. In cases 7, 9 and 12 the patients had obviously survived for many days after the suprarenal necrosis had occurred and death in each case was due to some intercurrent condition. If fortune had been more favourable presumably these three patients would have recovered, as their suprarenal lesions, though extensive, were not equally severe on both sides. Thus it seems reasonable to presume that there are some patients with suprarenal lesions of this type who do in fact recover. It is impossible to say how many such patients there are, as the suprarenal lesions at the initial stage cannot be diagnosed with certainty by present clinical methods. The subsequent course of the suprarenal lesions in the surviving patients can be prognosticated from ordinary pathological principles. Small cortical hæmorrhages would either be absorbed or heal with small scars ; the healing of the large areas of necrosis which involve the whole cortex would, however, result in a very thin cortex consisting almost entirely of fibrous tissue, possibly showing some round-cell infiltration and with occasional small areas of surviving cortical cells which would give rise to hyperplastic nodules. These appearances are in fact seen in a number of patients who die of Addison's disease not due to tuberculosis, as described by Barnard (1930), Guttman (1930), Hellier (1930) and Susman (1930.) The condition is sometimes referred to as cortical atrophy but this term is better kept for the true atrophy which occurs in hypopituitarism. Guttman, in a comprehensive review of 566 deaths due to Addison's disease, found that in 70 per cent. the destruction was due to tuberculosis, 19 per cent. were what he called "primary contracted suprarenals", and the remaining 11 per cent. were due to a variety of causes such as tumour, amyloid, etc. It is of interest that while he found males to predominate over females in the tuberculosis group by about 2 to 1, in the "primary contracted" group females predominated by 1·6 to 1. In both groups the maximum number of deaths occurred at the age of about 38 years. Susman gives detailed pathological findings of five cases of Addison's disease due to atrophy of the cortex of unknown ætiology and of these cases four were females and one male. In a second paper (Susman, 1936) dealing with the incidence of "atrophy" of the adrenals he finds that from results published in England 33 per cent. of cases of Addison's disease are due to "atrophy" while the results from other countries show about a 10 per cent. incidence. In the English group females predominated over males by almost 6 to 1 and all the females were aged between 36 and 46 years. Susman and Guttman both describe the "atrophy" of the cortex as of unknown ætiology. It appears probable that many of these "atrophic" suprarenals may have been the healed stage of some severe acute destructive lesion which occurred some time, perhaps years, previously. Further study is required to assess how many may have originated as non-fatal cases of the obstetric type described here or of the Waterhouse-Friderichsen syndrome. A term such as post-necrotic scarring of the suprarenals would be a more accurate description of the pathological state of these glands than the terms "primary contracted suprarenals" and "atrophy of the suprarenals" used by previous writers.

The suprarenal of a scarred-cortex case is shown in fig. 6. This woman had one pregnancy which was reported as normal but after delivery she was moved to an isolation hospital for puerperal pyrexia. She responded moderately well to treatment but died in a typical Addisonian crisis about two years later. At autopsy no suprarenal glands could be found but sections taken from the fat in the neighbourhood showed surviving suprarenal medulla, some masses of small round cells and a few surviving cortical cells (fig. 6).

## DISCUSSION

A study of the literature on suprarenal hæmorrhage and necrosis, excluding well-defined syndromes such as the Waterhouse-Friderichsen syndrome and the suprarenal hæmorrhage of infancy, reveals a remarkable complexity and variety of associated conditions. Hæmorrhage and focal necrosis in the suprarenal cortex have been reported in cases of cortical necrosis of the kidneys by Weaver and von Haam (1939), Jardine and Teacher, and Evans and Gilbert. Three of these cases (Jardine and Teacher, 2 cases; Evans and Gilbert, 1 case) occurred in pregnant women suffering from extremely severe eclampsia, while the case of Weaver and von Haam followed tonsillitis. The changes described in the suprarenals closely resembled the findings in some of the present series but cortical necrosis of the kidneys was not found in any of the present cases. Rich (1944) describes a focal necrosis of the suprarenal cortex in two non-pregnant patients with fulminating infection. Mitchell and Angrist (1943) describe focal necrosis of the suprarenal cortex without gross hæmorrhage in eighteen cases, mainly acute infections, and from the multiplicity of ætiological factors they conclude that there is a physiological factor in the production of the necrotic lesions.

Many of the cases so far reported have occurred in pregnancy. Arnold, Richer and Lepore found that five out of eighteen previously reported cases of suprarenal hæmorrhage were pregnant women and they consider that there must be an increased liability to the lesion during pregnancy. This is supported by the fact that there are now more than 20 recorded cases of suprarenal hæmorrhage associated with pregnancy in addition to the 14 cases reported here. The reason for this association is perhaps the co-incidence during pregnancy and the puerperium of the increased risks of metabolic deficiencies and of massive infection. Certainly these two factors, together or separately and in varying degree from case to case, appear prominent in the present series and also in the previously reported cases associated with pregnancy. The condition must be regarded as a similar phenomenon to the Waterhouse-Friderichsen syndrome but with different ætiological factors.

## SUMMARY

Fourteen cases of hæmorrhage and necrosis of the suprarenal cortex during pregnancy and the puerperium are reported. The histology of the lesions is described and the clinical aspects of the cases discussed. Massive infection and the toxæmias of late pregnancy associated with severe malnutrition appear to be possible ætiological factors.

Non-fatal cases may later develop Addison's disease and have severely scarred or almost non-existent cortices; they presumably form part of the group previously described as "primary contracted suprarenals" or "atrophy of the suprarenals," but more appropriately named post-necrotic scarring.

I am greatly indebted to Professor H. L. Sheehan and Professor T. Crawford for their generous help in the preparation of this paper. Thanks are also due to Dr Lederer, Doncaster Royal Infirmary, for the notes and material from the case of Addison's disease.

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### Appendix

### SUMMARIES OF CASE REPORTS

*Case* 1 Aged 36 years, 5th pregnancy She developed œdema and at 28th week had sudden severe back pain One hour later she became comatose and died in 3 hours without being delivered Autopsy showed evidence of malignant hypertension with gross eclamptic lesions in the liver and a large cerebral hæmorrhage Both suprarenal glands showed multiple patches of hæmorrhage deep in the cortex but no necrosis Venous thrombosis and poly morphonuclear infiltration were not present but there was marked round cell infiltration The lesions were judged only a few hours old

*Case* 2 Aged 25 years, 4th pregnancy She developed œdema, anorexia and asthenia during the last month of pregnancy One week before term she became suddenly blind and was delivered spontaneously after 6 hours labour She collapsed and 10 hours after delivery had 3 fits and became comatose (Temp 102° 106° F , Pulse 160/min , Blood urea 112 mg /100 ml , Blood uric acid 14 mg /100 ml ) She was given 150 ml of 50 per cent glucose solution intravenously but died 42 hours after delivery Autopsy showed broncho pneumonia, eclamptic lesions in liver and kidney and petechial hæmorrhages in the brain and endocardium The suprarenal glands showed gross distension of the central veins and cortico medullary regions with hæmorrhage radiating into the outer cortex There was some necrosis and absorption of the cortical cells in the most congested areas but elsewhere the cells appeared normal The R B C in the deeper layers of the cortex showed loss of hæmoglobin There was no pigment and polymorphs were scanty The lesions appeared of about 36 hours' duration

*Case* 3 Aged 35 years, 5th pregnancy She had no antenatal care but developed some œdema and was delivered spontaneously at 40 weeks She collapsed 5 hours later and had 2 eclamptic fits Death occurred 41 hours

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# CYLINDROMATOUS MUCOUS-GLAND TUMOURS OF THE TRACHEA AND BRONCHI: A REPORT OF THREE CASES

R. H. R. Belsey and J. C. Valentine

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(Plates LVI-LVIII)

Reisner in 1928 described a benign epithelial tumour of the bronchus and drew attention to other possible cases in the German literature. Since then there have been many and full accounts of such tumours by American, British and Scandinavian authors (Wessler and Rabin, 1932; Jackson and Konzelmann, 1936-37; Foster-Carter, 1941; Engelbreth-Holm, 1944-45; Moersch *et al.*, 1945; Holley, 1946). These tumours are usually referred to as adenomas and consist of solid masses of cells of remarkably uniform appearance supported by a scanty vascular stroma and often having an acinar arrangement.

There is, however, another epithelial tumour of the trachea and bronchi which runs a relatively benign course, but which, although in many respects resembling the commoner adenoma, differs greatly from it in its histological appearance. This is the so-called cylindroma. This term was originally coined by Billroth (1856, quoted by Kramer and Som, 1939) to describe a tumour of the orbit in which there were intertwining cylinders of epithelial cells in a hyaline stroma. Since Billroth's original description the term has been much abused and in 1877 Heschl pointed out that there were three different types of cylindroma and then added a fourth: this was a connective tissue tumour of the lung and appears to have little relationship to the cylindroma described by Billroth. Cylindroma of the lung has been adequately described in America (Kramer and Som, 1939; McDonald *et al.*, 1945) but has received little attention in the British literature and we have found records of only four cases. Cann (1938) reported two basal-cell carcinomas of the trachea which were substantially the same as the tumours here described. Foster-Carter's case 16 and case 6 of Willis's (1948, p. 379) collection of pulmonary tumours are also of this type. In view of this virtual omission from the British literature we wish to report three such cases which have come under the care of one of us (R. B.) in the last 7 years.

## CASE REPORTS

### Case 1

#### *Clinical history*

R. P., a man aged 54 years, was admitted with a history of eight attacks of right-sided pneumonia during the previous two years. Cough and expectoration of blood-streaked sputum had persisted for the last seven months. There was collapse of the right middle and lower lobes. Bronchoscopy showed complete obstruction of the right stem bronchus just below the upper lobe orifice by a tumour having the macroscopic appearance of the polypoid type of bronchogenic carcinoma. As much as possible of the tumour was removed with biopsy forceps to permit drainage of the obstructed segments of lung tissue. Histological examination of these fragments failed to show whether the "tumour" was neoplastic or inflammatory. Following partial removal of the tumour the right middle lobe re-expanded. A right pneumonectomy was performed and a post-operative course of X-ray therapy to the chest was given. When seen three months later he was well, but four and a half months after the operation he died at home and no autopsy was performed.

#### *Pathological examination of the tumour*

The original bronchoscopic biopsy contained fragments of bronchial mucosa beneath which were what appeared to be dilated mucous glands and it was thought that this might be due to inflammatory reaction. Later, when the sections of the tumour were examined, it became clear that what had been thought to be dilated mucous glands were in fact part of the tumour and it should have been possible to diagnose the condition from the original biopsy.

The tumour measured 5 cm. in its greatest diameter. It arose by a broad base from the main bronchus of the right lower lobe and projected into the lumen in polypoid fashion towards the trachea for a distance of 2·5 cm. This extension was blunt and presented on the whole a smooth appearance, but closer examination showed it to have a mammilated surface. There was a similar but much smaller extension of the growth peripherally which completely blocked the main bronchus. Beyond this obstruction the bronchus was dilated and filled with thick yellow pus. The tumour was firm and elastic and varied in colour on section. In places it was homogeneous and white, with fine fibrous trabeculæ: other portions were almost gelatinous in appearance. Although the tumour was for the most part well circumscribed and appeared to be compressing rather than infiltrating the surrounding structures, invasion was evident in places. A hilar lymph node was much enlarged and contained obvious tumour tissue of precisely the same macroscopic appearance as the tumour in the bronchus.

Histologically the bronchial epithelium is intact over much of the tumour but in places has been destroyed by inflammation. At one point exuberant granulation tissue has burst through the epithelium to enter the lumen of the bronchus. The tumour has a characteristic pattern (figs. 1 and 2) consisting of masses of epithelial cells surrounding

CYLINDROMATOUS MUCOUS GLAND TUMOURS

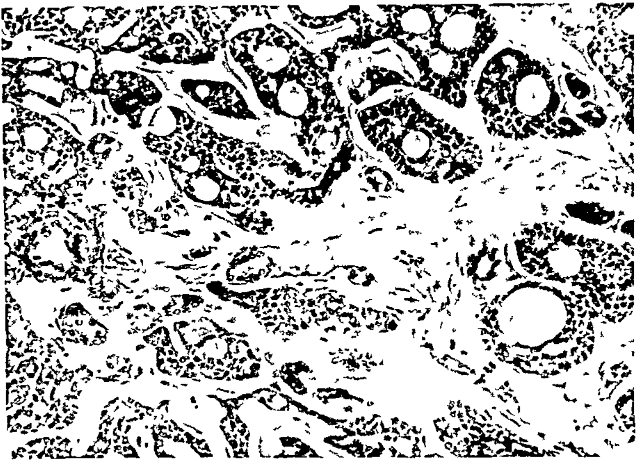

FIG 1.—Case 1 General histological appearance of tumour, showing prominent hyaline stroma Hæmatoxylin and eosin ×200

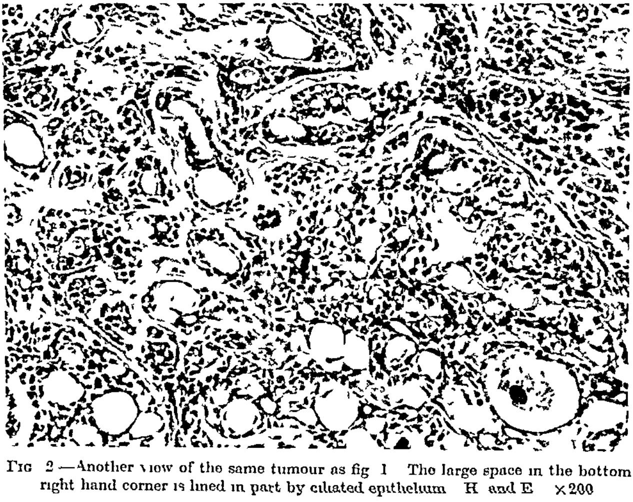

FIG 2.—Another view of the same tumour as fig 1 The large space in the bottom right hand corner is lined in part by ciliated epithelium H and E ×200

spaces which are usually filled with homogeneous eosinophilic material. These spaces vary from five to several hundred microns in diameter. Serial section shows that many of them are tubular in shape and that they communicate with one another. The cell masses are set in a hyaline or mucoid stroma, in addition to which there is a loose and rather inconspicuous fibrous tissue framework with occasional smooth muscle cells. The amount of mucoid material varies greatly, being scanty in the cellular areas while in other places it is contained within large spaces lined with flattened or cubical epithelial cells, the appearance being not unlike that of a colloid goitre (fig. 3). In some places the amount of mucoid stroma is greatly increased so that a lacework pattern is produced in which the epithelial cells are greatly distorted, becoming stellate or elongated. This has proceeded to such a degree that in some places the tumour is largely composed of mucoid material with isolated clumps or single epithelial cells enmeshed within it (fig. 4). This gives rise to an appearance closely resembling that seen in "mixed" parotid tumours. In yet other places the epithelial cells at the centre of the cell masses are arranged in a loose network while those at the periphery are lined up in palisade fashion like an adamantinoma or basal-cell cancer of the skin.

The tumour tissue generally is well circumscribed and contained within a fibrous-tissue capsule produced by condensation of the surrounding lung tissue. There is, however, definite neoplastic invasion of bronchial cartilages, lung tissue and perineural lymphatics. An intrapulmonary lymph node as well as the hilar node mentioned previously also contains tumour.

In the more cellular areas the size and shape of the cells are variable, being either round, spindle-shaped or triangular. The nuclei are usually small and darkly staining, though some are paler and these contain a fine evenly scattered chromatin network and often a single nucleolus. Mitotic figures are generally few, but in places, particularly where the cells are closely packed, as many as three per high-power field are found.

Some of the spaces are lined by cells which differ from the usual in being larger, their cytoplasm in particular being quite abundant. The nuclei are rounder and more pale-staining but the most striking feature is that a number possess well-developed cilia at their free margin (fig. 5). Such cilia are found in spaces as small as 20 $\mu$ in diameter. Occasionally one finds spaces such as the one illustrated in which one side is lined with ciliated epithelium while the opposite side is lined with flattened cells which shade off imperceptibly into the ordinary tumour tissue. We believe that the ciliated epithelium is part of the tumour.

Several methods for staining mucin were used and gave strongly positive results in places, but negative results in others. The staining is usually more strongly positive in the larger spaces. No argentaffin granules are demonstrated in any of the tumour cells.

## Case 2

### *Clinical history*

F. M., a woman of 41 years, was admitted in 1943 with two years' history of dyspnœa, and discomfort in the right chest. There had been a hæmoptysis just before admission. The patient appeared well and there were no abnormal physical signs, but X-ray examination of the chest suggested collapse of an azygos lobe at the apex of the right lung. Bronchoscopy revealed a tumour with the smooth polypoid appearance characteristic of a benign adenoma. It was growing into the right main bronchus from the right-hand wall and almost obstructing its lumen. A biopsy was reported as benign adenoma of bronchus.

At operation the tumour was found to extend through the wall of the bronchus, obstructing the branch to the azygos lobe and extending upwards to involve the lower end of the trachea for half a centimetre above the level of the carina. A right pneumonectomy was performed and the infiltrated portion of the tracheal wall resected.

The patient remained well for six years but was then re-admitted with dyspnœa and stridor. Bronchoscopy showed an extensive recurrence of the tumour, which was now obstructing the lower end of the trachea and extending upwards to involve the greater portion of the intrathoracic segment of the trachea. The tumour was very vascular. A biopsy showed the same appearance as before. Extensive resection and reconstruction of the intrathoracic trachea was performed but it was not possible to remove an extension of the tumour down the medial wall of the left main bronchus. There was no evidence of lymphatic spread. Early convalescence was complicated by obstruction in the reconstituted trachea but this was relieved by bronchoscopy and the patient is now, 18 months after the operation, well, except that mild stridor is still present.

### *Histology*

The original biopsy sections were available for study. They show a picture identical with that of case 1 except that no ciliated epithelium is found in any of the sections. As in the first case, perineural lymphatic invasion is a prominent feature and in places mitotic figures are numerous. Further material from the recent recurrence shows precisely similar appearances, including prominent lymphatic permeation (fig. 6), and there is also invasion of the bronchial cartilage. The mucous membrane is still intact over the surface of the tumour (fig. 7).

## Case 3

### *Clinical history*

D. C., a woman of 42 years, was admitted in 1944 complaining of wheezing and a non-productive cough. Two years previously a "cyst" was said to have been removed from the trachea through the bronchoscope and the patient received a course of X-ray therapy lasting three months.

She was emaciated and stridor was marked. Bronchoscopy revealed a tumour with the typical appearance of a bronchial adenoma almost completely obstructing the lower third of the trachea. A biopsy showed a benign tracheal adenoma. There was no evidence of metastasis.

A right transpleural thoracotomy was performed and a portion of tracheal wall bearing the tumour and its base was excised and the resulting defect

CYLINDROMATOUS MUCOUS GLAND TUMOURS

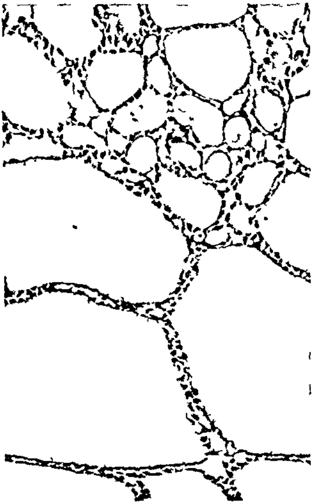

FIG 3 —Case 1 Greatly dilated spaces filled with mucoid material which stains variably with mucin stains H and E 200

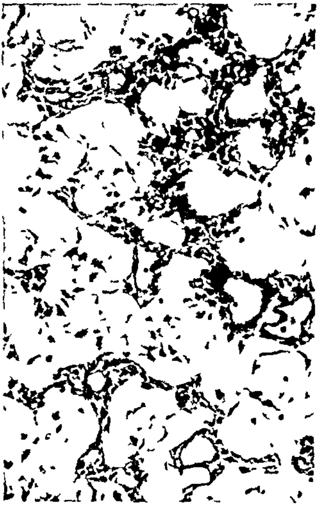

FIG 4 —Case 1 Great increase of the mucoid matrix surrounding islets of epithelial cells and producing a picture typical of mixed salivary gland tumour H and E ×200

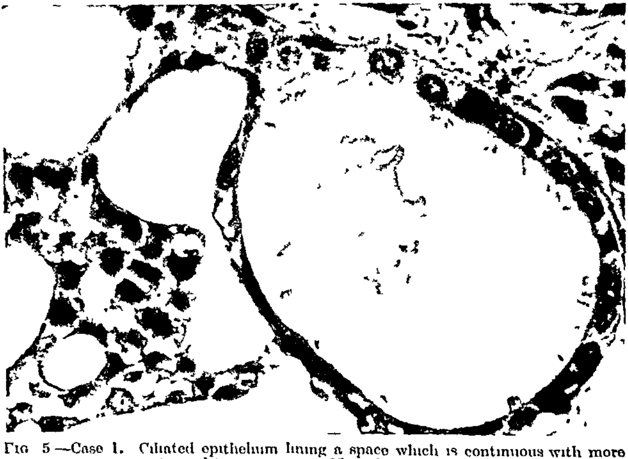

FIG 5 —Case 1. Ciliated epithelium lining a space which is continuous with more typical tumour tissue H and E ×800.

CYLINDROMATOUS MUCOUS-GLAND TUMOURS

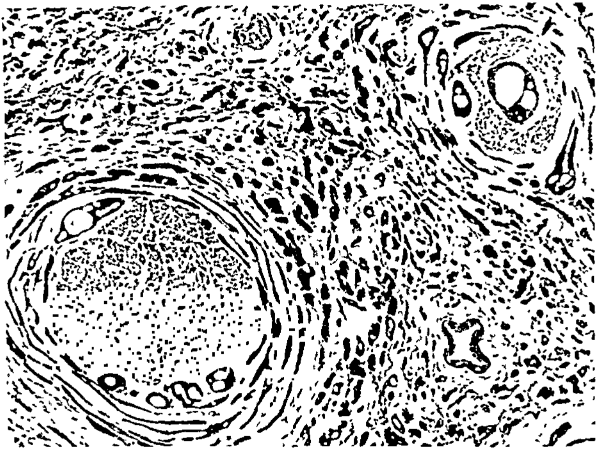

FIG. 6.—Case 2. Invasion of perineural lymphatics and of a nerve bundle (top right-hand corner) by tumour. H. and E. ×200.

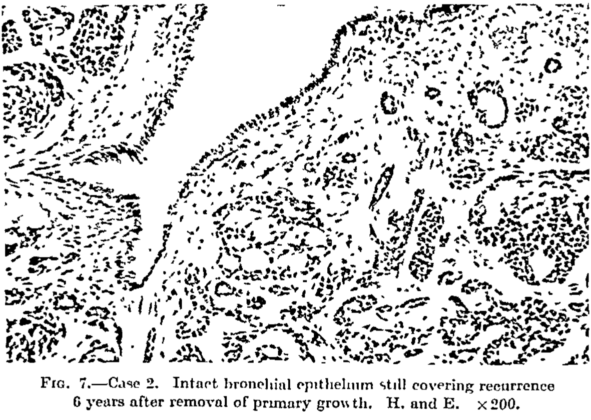

FIG. 7.—Case 2. Intact bronchial epithelium still covering recurrence 6 years after removal of primary growth. H. and E. ×200.

repaired. Convalescence was uneventful and the patient's symptoms were relieved.

A year later she was readmitted with recurrence of the tumour at the same site and a return of wheezing and cough. Biopsy showed the same appearance. The stridor became rapidly worse and an extensive resection of the intrathoracic trachea with plastic reconstruction was carried out. The growth was found to infiltrate the tracheal wall extensively from the level of the carina to a point about one inch below the apex of the thorax, but had not encircled the lumen. The patient was again relieved of all symptoms for a year but then relapsed and died. At autopsy there was further recurrence in the right main bronchus and upper part of the intrathoracic trachea; there were also seedling nodules in the right pleura, probably implanted at the time of the second operation.

### *Histology*

Sections from the original biopsy and a portion of the pleura removed at autopsy were available for study. These show precisely the same appearances as those of case 2, but in this instance lymphatic permeation is found only in the specimen of pleura.

## DISCUSSION

### *Morbid anatomy and histology*

There is little in the naked-eye appearance which serves to distinguish the cylindroma from the ordinary adenoma unless it be the slightly glassy or gelatinous appearance which they possess and their tendency to involve the trachea more commonly than the bronchus, which the adenoma rarely does (McDonald *et al.*). When they do arise in a bronchus it is almost invariably a large one and a polypoid tumour is formed, the portion projecting into the lumen being directed towards the trachea, due, it is thought, to the convulsive efforts of coughing (Kramer, 1930; Schwartz, 1944). They arise by a broad base and the extra-bronchial portion may be of considerable size. The tumour is usually sharply demarcated from the rest of the lung but may show local invasion as in our first case.

A characteristic feature which these tumours share with the ordinary adenoma is the fact that the bronchial epithelium is usually intact over the surface of the growth. Not uncommonly, however, there is squamous metaplasia and the mucosa may become greatly thinned, as in our first case, where it was reduced to a single layer of greatly flattened cells.

Most authors stress the absence of mitoses and yet in all our cases they were numerous in places. Clerf and Bucher (1942) also mention abundant mitoses but do not make it clear whether there was any difference in this respect between their solid and their cylindromatous types.

It has been stated that the presence of mucus in these tumours is a characteristic feature differentiating them from the solid type of bronchial adenoma, but this is not so, since there are a number of instances in the literature of solid adenomas with mucus production (Gowar, 1936-37 ; Foster-Carter, 1941 ; Engelbreth-Holm, 1944-45). The variation in the staining reactions of the mucoid material which we have noted has also been recorded by others (McDonald *et al.*, 1945) and Lemaitre (1938) has pointed out that mucicarmine staining is not always typical, even in normal mucous glands.

## *Histogenesis*

Since both solid and cylindromatous types are commonly covered by intact bronchial epithelium it is unlikely that they arise from it. The only other epithelial structures in the bronchus and trachea are the mucous glands and their ducts, and most authorities are agreed that these are the probable source of these tumours. Fried (1934) and Laff (1940) state that they arise only in bronchi whose diameter is greater than 10 mm. and suggest that the explanation for this is that mucous glands are not found in the smaller bronchi. This is incorrect ; Maximow and Bloom (1942) state that glands can be found as far out in the bronchial tree as the cartilage extends, that is, to bronchi of about 1 mm. diameter. That adenomas can arise peripherally has been shown by Mayo (1942) and by Maier and Fischer (1947), but they are extremely rare and it is difficult to explain why this should be so. It is conceivable that they do not give rise to symptoms in the smaller bronchi and so do not come under observation at an early stage. This suggestion receives some support from Maier and Fisher, who found that in five patients with adenomas arising in branch bronchi two were apparently well when the tumour was detected by mass radiography. On the other hand, one would expect to see occasional cases at autopsy and so far as we are aware none has been reported.

Since these tumours are almost certainly derived from the mucous glands of the bronchus and trachea one would expect them to be similar in histological appearance to tumours of the salivary glands, and this is indeed the case. Harvey *et al.* (1938) in their discussion of salivary-gland tumours state that similar tumours can occur in the bronchi, and their photomicrographs of salivary-gland tumours are in a number of instances identical with those of our cylindromatous tumours of the trachea and bronchus. A similar resemblance is found in the illustrations of Patey (1930-31). Engelbreth-Holm, while admitting the striking resemblance between these two groups of tumours, points out that there are several features characteristic of the " mixed " salivary-gland tumour which are not found in the bronchial adenomas of either solid or cylindromatous type. One does not, for instance, see the chondroid material which is such a feature of the salivary gland tumour. It is true that the classical chondroid material is not seen but myxomatous changes can be found in our first case (fig. 4) and are also illustrated by Kramer and Som. By no means all salivary-gland tumours contain chondroid material and in a series of 81 such tumours examined by Dockerty and Mayo (1942) 15 presented a purely cylindromatous appearance. Lemaitre also has reported purely cylindromatous tumours of the salivary glands.

Bone and cartilage are not infrequently found in bronchial adenomas of the solid variety and most authors attach little importance to this finding, regarding them as either inclusions of normal bronchial cartilage or as evidence of metaplasia on the part of the stroma (Stout, 1943 ; Holley, 1946). We agree with this view. Cartilage when present is usually normal bronchial cartilage involved in the tumour. Bone formation too is readily explained, since it is not uncommonly found in the lung in healed tuberculosis and may sometimes be found in mitral stenosis (Lawson, 1949). The contention of Womack and Graham (1938) that these tumours are true mixed tumours in the sense that they are derived from more than one germinal layer is, we feel, unjustified, and the fact that they resemble " mixed " salivary-gland tumours in no way strengthens the argument, since most authorities are agreed that they also are entirely epithelial in origin (Harvey *et al.*, 1938 ; Willis, 1948, p. 322).

One is struck by the histological resemblance of parts of these tumours to some types of rodent ulcer, and it was this among other things which led Cann to classify his two tracheal tumours as basal-cell carcinomas, a view that is supported by Beck and Guttman (1936), Kramer and Som (1939) and Engelbreth-Holm (1944). Harvey *et al.* draw attention to Krompecher's observation that many of the tumours of salivary and lachrymal glands resemble rodent ulcers and Dockerty and Mayo found evidence that salivary-gland tumours are derived from basal cells. Harvey *et al.* themselves suggest that salivary-gland tumours are derived from a primitive replacement epithelium which may form gland acini or ducts or may become neoplastic. The tumours so formed vary in appearance within the limits set by the potentialities of the primitive cell. This being so, the argument in the literature as to whether these tumours are derived from the ducts or the gland itself becomes pointless. Origin from primitive replacement epithelium would also explain the presence of ciliated epithelium in our first case.

Opinions vary as to whether these tumours are to be regarded as separate from the ordinary adenomas. McDonald *et al.* separate them sharply on both clinical and histological grounds but Anderson (1942-43) and Willis (1948, p. 382) regard them as fundamentally the same. Engelbreth-Holm found two tumours in which areas characteristic of both types could be found. In our three cases the histological appearances are quite distinct from those seen in the ordinary adenoma but we feel that this argument too is resolved by the suggestion that these tumours are derived from primitive replacement epithelium, and that while in most instances the two tumours are histologically distinct they have a recent common ancestry and mixed types may occur.

### *Malignancy and prognosis*

Whatever the histogenesis of these tumours they must not be confused with the more common bronchogenic carcinoma, the prognosis of which is so much worse (Churchill, 1939). The majority are of low-grade malignancy and carry a more favourable prognosis than any of the usual bronchogenic carcinomata. This is well illustrated by Fried (1947), who reported only 3 of 28 patients alive and well 5 years after surgical excision of carcinoma of the lung, while many of the reported cases of cylindroma have a history of many years of bronchial obstruction prior to surgical treatment or biopsy. Nevertheless, there is an increasing number of cases in the literature of bronchial adenoma and cylindroma in which there is undoubted evidence of malignancy (table), and it is now clear that these tumours are by no means as benign as was originally believed.

TABLE

*Cases collected from the literature in which metastases have been reported*

| Author | Site of secondaries | Type of tumour |
|---|---|---|
| Chamberlain and Gordon (1945) | Same lung | ? Cylindroma |
| Laff and Neubuerger (1944) | Other lung | Cylindroma |
| Womack and Graham (1938) | 1. Liver and lung | Adenoma (no histology of metastases) |
| | 2. Liver | Adenoma |
| Anderson (1942-43) | Liver | Cylindroma |
| Holley (1946) | 1. Liver | Adenoma |
| | 2. Kidney | Cylindroma |
| Willis (1948, p. 379) | Lungs, pleura, mediastinum, liver, vertebræ, subcutaneous tissue | Cylindroma |
| Adams, Steiner and Bloch (1942) | 1. Lumbar vertebræ | Adenoma |
| | 2. Liver, lymph-nodes | Adenoma |

Lymph-node involvement has also been reported by a number of authors, including Adams *et al.* (1942), Chamberlain and Gordon (1945) and Crafoord and Lindgren (1945).

A further manifestation of malignancy, noted by Anderson and illustrated by Engelbreth-Holm, is the tendency to invade blood vessels and perineural lymphatics. It is of interest in this connection that Dockerty and Mayo found that the cylindromatous type of salivary-gland tumour shows a greater tendency to invade the perineural lymphatics than other varieties. Apart from lymphatic and blood-vessel permeation, local tissue invasion is regarded by McDonald *et al.* as a distinguishing feature between cylindroma and adenoma, but other writers are less certain of this (Clerf and Bucher ; Holley). We support the view of McDonald *et al.* and believe that the cylindromatous tumour is distinctly the more invasive of the two. This opinion is based upon the histories of 13 cases of ordinary adenoma seen between 1943 and 1948. One of these died as a result of operation and one recurred after inadequate removal. All the remainder are well.

Another feature of these tumours is their great tendency to local recurrence, well illustrated in our second case. In a personal communication Dr Edith Dawson mentions a tumour of this type which recurred 3 or 4 times after local removal. The resemblance to "mixed" salivary-gland tumours is again evident in this tendency to recur locally but to metastasise rarely.

It is clear, therefore, that apart from their secondary effects on the lung these tumours are by no means entirely benign and must be regarded as carcinomata of low-grade malignancy. Some discussion will be found in the literature as to whether they ever become converted into the more usual types of bronchogenic carcinoma. It seems extremely unlikely that this ever occurs, since one does not see intermediate types and the sex incidence is so very different. Bronchial adenomas are at least as common in women as in men. Of 120 cases reviewed by Foster-Carter 62 per cent. occurred in women. This is in marked contrast to the figures given for bronchogenic carcinoma, of which 75-90 per cent. occur in men (Brunn and Goldman, 1941; Hadfield and Garrod, 1947).

## *Classification*

It has been shown that, judged by their behaviour, these tumours must be regarded as carcinomata. In so doing, however, they must not be confused with the more usual types of bronchogenic carcinoma, nor must they be confused with the ordinary adenoma of the bronchus, for although they have a common origin they are histologically distinct and considerably more malignant.

Some pathologists object to the term cylindroma as being ambiguous and uncertain in its meaning and Willis (1948, p. 336), in discussing salivary-gland tumours says of the term that "... it has no real meaning save as a nickname for a certain type of structure, and it is used in different senses by different writers". If the word is to be rejected what then is to take its place? It seems scarcely justifiable to introduce yet another term and in recent years at least the histological descriptions of cylindromas of the trachea and bronchus have been quite uniform. We therefore suggest that the word—whatever its shortcomings—be retained as a descriptive title, that these more lowly malignant tumours of the trachea and bronchi be classified as mucous-gland tumours and that they be subdivided into (1) adenoma (2) cylindroma, and (3) a mixed type.

## SUMMARY

1. Three cases of a cylindromatous type of tumour of the trachea and bronchus are described, in one of which ciliated epithelium was present as part of the neoplastic process.

2. The literature is reviewed and a similarity traced between these tumours, salivary-gland tumours and basal-cell carcinomas of the skin.

3. It is suggested that they be classified as a sub-group of the mucous-gland tumours of the trachea and bronchi, and that they be separated from the more usual type of adenoma of the bronchus, to which they are related by a common ancestry from primitive replacement epithelium.

4. Cylindromas of the trachea and bronchi behave as carcinomata of low-grade malignancy, with a pronounced tendency to local recurrence. They rarely metastasise, but invade locally and are rather more malignant than the ordinary adenoma.

We wish to thank Prof. T. F. Hewer for encouragement and criticism in the preparation of this paper and Dr O. C. Lloyd for helpful suggestions. The photomicrographs were taken by Mr G. H. Rogers.

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(2) Decolorise with 70 per cent. ethyl alcohol until the blue colour remains in the chief cells only.

(3) Wash with water.

(4) Stain with phloxine 2 per cent. aqueous solution for 3 minutes.

(5) Wash with water.

(6) Stain with methyl green 1 per cent. aqueous solution for 30 seconds.

Result :—Pepsinogen granules purple ; parietal cell cytoplasm and Paneth granules bright red ; nuclei and goblet-cell mucus green.

### *Clinical material*

The biopsy procedure was carried out on 150 patients complaining of a variety of dyspeptic symptoms and in whom an opaque meal showed no gross abnormality. Forty-three cases of pernicious anæmia were also investigated.

## NORMAL HISTOLOGY OF THE BODY MUCOSA

The normal mucosa of the body of the stomach consists (fig. 1) of a mucus-secreting surface epithelium dipping down into foveolæ. Into each foveola several tubules open. The necks of these tubules are lined by mucus-secreting cells, then follow parietal and chief cells. The parietal cells are situated mainly in the upper one-third of the tubule, while a few are applied to the outside of the chief cells in the lower two-thirds.

In the 14-weeks-old embryo gastric glands can be seen beginning to differentiate from foveolæ. In the adult the epithelial cells of the foveolæ show frequent mitoses (Bensley, 1928). Thus it seems probable that replacement of the tubular epithelium, even in adult life, is by the same method as its original formation in the embryo. There is also an obvious gradation in differentiation from the cells in the depths of the foveolæ to the fully differentiated mucus-secreting surface cells, indicating that replacement of these cells is also by foveolar cell multiplication. This becomes more obvious during the replacement of surface epithelium following its loss from injury (fig. 4).

## HISTOPATHOLOGY OF THE BODY MUCOSA

No significant change is seen in the gastric mucosa of fifty-five of the cases of dyspepsia. In the remainder, various degrees of atrophy of chief and parietal cells are present. In these patients the severity of the atrophy is in general proportional to the extent of the gastritis, so that two broad classes can be recognised, namely :—

1. Superficial gastritis with minimal atrophy (figs. 2 and 3)—45 cases.
2. Gastritis extending into the depths of the mucosa with moderate to severe atrophy—50 cases.

In the 43 cases of pernicious anæmia there is a range of almost complete to complete atrophy of chief and parietal cells but gastritis is absent or minimal.

## GASTRITIS AND GASTRIC ATROPHY

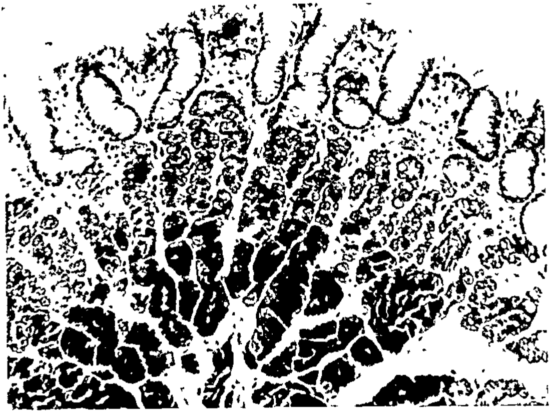

FIG. 1—Normal gastric (body) mucosa. The cytoplasm of the mucus-secreting cells is unstained while the parietal cells are lightly stained, with obvious intracellular canaliculi. Pepsinogen granules in the chief cells are darkly stained. Trichrome. ×150.

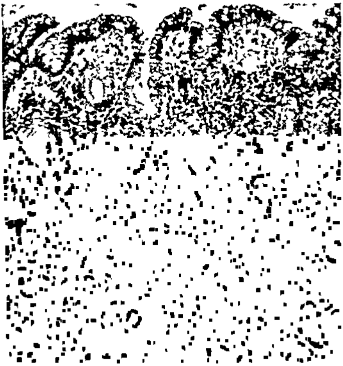

FIG. 2.—Superficial gastritis, showing lengthening and tortuosity of the pits and infiltration of the superficial zone of the lamina propria by wandering cells Hæmatoxylin and eosin. ×120.

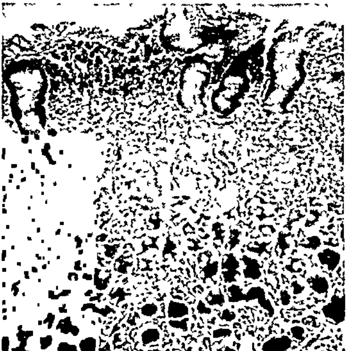

FIG. 3—Superficial gastritis. The same specimen as fig. 2 stained to show the normal basal two-thirds of the tubules, with plenty of dark-staining pepsinogen granules in the chief cells. Trichrome. ×120.

GASTRITIS AND GASTRIC ATROPHY

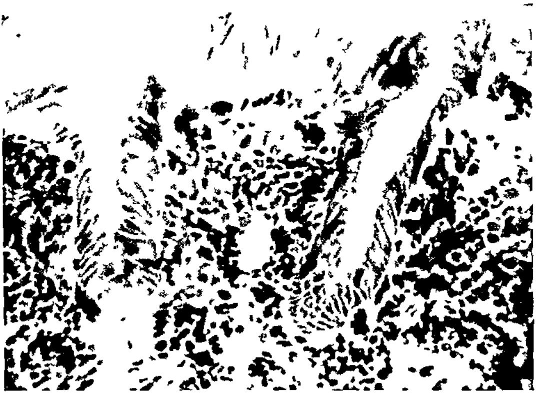

FIG 4 —Gastric mucosa three days after hæmatemesis following a severe alcoholic bout The necrotic surface epithelium is being replaced by actively proliferating epithelial cells growing out from the pits The lamina propria shows many darkly stained extravasated red cells Trichrome ×400

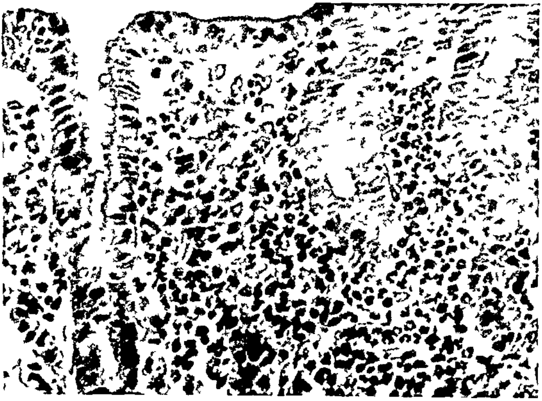

FIG 5 —Superficial gastritis, showing flattened surface epithelium with many polymorphs in the superficial zone of the lamina propria Hæmatoxylin and eosin ×400

So far biopsies have been repeated in only a few cases, and so no attempt was made to assess the acuteness or chronicity of any gastritis observed.

### *Superficial gastritis*

The morphological changes constantly noted in this group are as follows:—The surface epithelium is flattened and often forms a syncytium more than one cell thick, with indistinct cell borders and hyperchromatic nuclei. *This appearance is one of recent epithelial* regeneration, occasionally plainly beginning in the foveolæ (fig. 4). The function of mucus secretion is poorly developed in these cells. That shedding of the surface layer had occurred is shown by patches which are completely denuded of the mucus-secreting cells, and in three cases investigated soon after excessive alcoholic intake, necrotic clumps of these cells are seen in the adherent mucus. Regenerating epithelium of similar appearance is seen experimentally in the healing of the mucosal breach produced in the cat's stomach by biopsy (Gunter, 1950).

There may be rupture of the capillary plexus, which is immediately subjacent to the surface epithelium, with extravasation of blood into the stromal tissues. Occasionally, as in acute alcoholism, hæmatemesis has resulted. The superficial portion of the lamina propria shows dense infiltration with wandering cells, predominantly neutrophil polymorphs (fig. 5), but lymphocytes and plasma cells are also present in varying numbers. The plasma cells occupy a more superficial position than the lymphocytes. There is evidence of active multiplication of the foveolar cells, causing a dilatation and lengthening of the pits (fig. 2). Mucus-secreting neck cells extend down the tubules. This may be due either to a failure in the normal differentiation into pepsin-secreting cells or to an actual change from pepsin secretion to mucus secretion in cells already differentiated (fig. 6). Change from one type of cell to another is a commonly observed phenomenon. Harvey (1906-07) noted that after gastroenterostomy in dogs the pepsin-secreting chief cells change to mucin-secreting cells for a distance of 7 mm. from the line of the anastomosis. This change was complete in three weeks. Florey *et al.* (1932) also observed that serous-secreting cells in the trachea of the cat change to the mucus-secreting type under the influence of various irritants.

In superficial gastritis, the basal two-thirds of the tubules remain normal (figs. 2 and 3). Here the parietal cells stain well and the chief cells contain plentiful pepsinogen granules. Histamine test meals in these patients show low or absent acid and a reduction in pepsin values. If the damaging agent can be determined, as in the alcoholic, and removed, it is found that in approximately 2-3 weeks the mucosal appearance has returned to normal. Similarly, in dogs subjected to gastroenterostomy (Harvey), in six months the region of the anastomosis again shows normal gastric tubules.

### *Gastritis with moderate to severe atrophy*

Here the full length of the tubule may become lined by mucus-secreting cells, forming a pseudopyloric gland. More often the base still shows both chief and parietal cells. There is always a histamine-fast achlorhydria with low pepsin secretion in patients whose biopsy shows these changes. The active proliferation of the foveolar cells produces tortuosity in the pits, so that they are often seen in transverse section. The surface epithelium is flattened but occasionally shows small bud-like syncytial masses. Neutrophil polymorphs are numerous close to the surface and around the pits. They can be seen surrounded by a small clear space in various stages of migration through the limiting epithelium (fig. 7). Plasma cells are plentiful and in approximately one-half of the specimens lymphocytes are aggregated into follicles near the base of the mucosa. In two specimens, adipose tissue is present, having probably developed by involution of these follicles (figs. 8 and 9). The muscularis mucosæ is thickened and its component muscle strands are separated and projected between the glandular elements to a varying extent. No specimen shows the gross hyperplasia of surface epithelium and pits described by Schindler (1947) and termed by him hypertrophic proliferative gastritis. The depth of mucosa was reduced to two-thirds to three-quarters of normal. Small patches of intestinal epithelium are present in about one-half of the cases showing severe mucosal atrophy. Intestinal epithelium is recognised by the presence of a striated border and of goblet and Paneth cells (fig. 12) at the base of the new tubules, while argentaffin cells are much more numerous than normal. Thus the altered conditions in the inflamed mucosa cause the foveolar cells to develop along lines which are abnormal for the stomach but normal for the small intestine. The end-result is the development of tubules indistinguishable from crypts of Lieberkuhn. This metaplasia is patchy, and abrupt changes from gastric to intestinal type can be seen in a single high-power field (fig. 10).

### *The mucosa in pernicious anæmia*

In the specimens obtained from these cases, thinning of the mucosa is invariable and often of extreme degree. In 30 per cent. it was reduced to one-half its normal depth. Chief and parietal cells are absent in 90 per cent. of samples, the tubules being lined by mucus-secreting neck cells or intestinal-type epithelium (fig. 11). Histamine-fast achlorhydria and insignificant peptic activity are obviously the results of these changes.

In pernicious anæmia it is rare to find any evidence of gastritis (fig. 11). A few lymphocytes and plasma cells are present in 50 per cent. of specimens, but only one case shows polymorphonuclear neutrophil leucocytes. The surface epithelium in pernicious anæmia

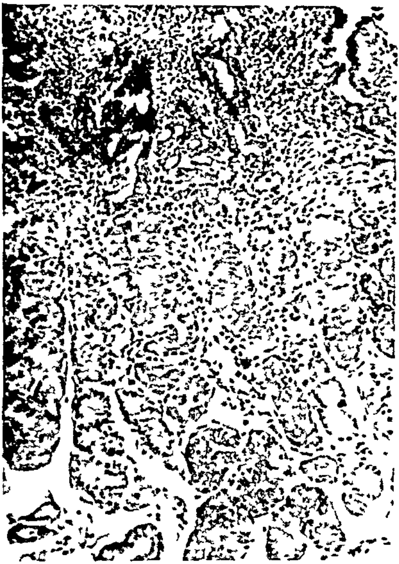

FIG 6—Superficial gastritis showing numerous wandering cells in the superficial zone of the lamina propria lengthening of the pits and a change of the two central tubules to mucin secretion as the lining chief cells now stain darkly with mucicarmine Hæmatoxylin and mucicarmine ×140

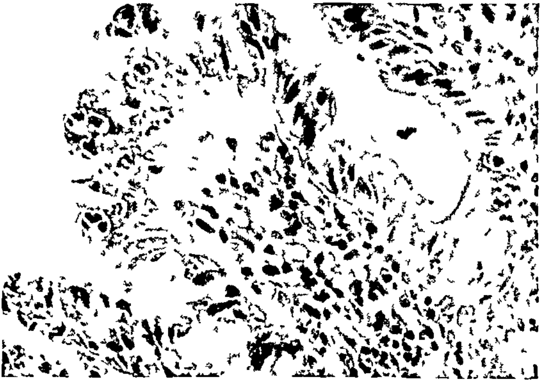

FIG 7—Gastritis with moderate atrophy View of the surface epithelium to show polymorphs, each surrounded by a clear space, migrating through the surface epithelium There is œdema of the villus near its tip Hæmatoxylin and eosin ×400

GASTRITIS AND GASTRIC ATROPHY

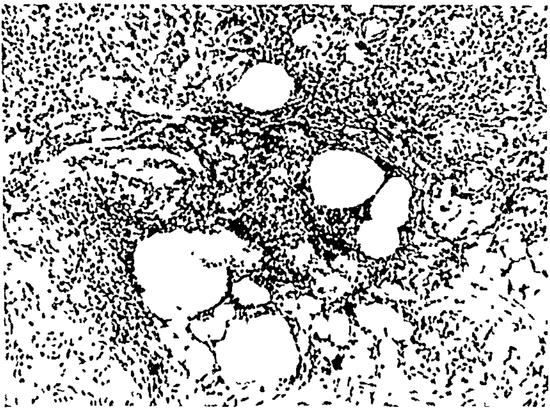

FIG 8.—Gastritis with severe atrophy of tubules, showing adipose tissue associated with a lymphoid collection. Hæmatoxylin and eosin. ×150

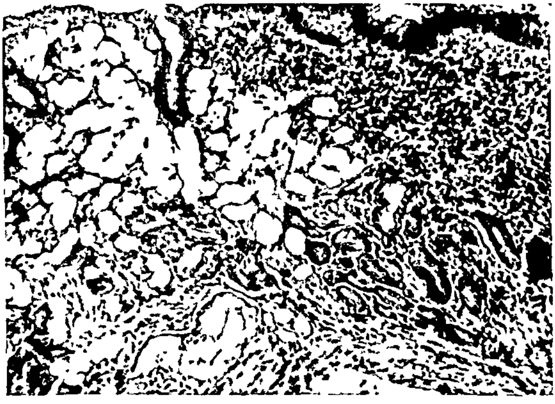

FIG. 9.—Gastritis with severe atrophy of tubules, showing development of adipose tissue within the mucosa. Hæmatoxylin and eosin. ×140.

GASTRITIS AND GASTRIC ATROPHY

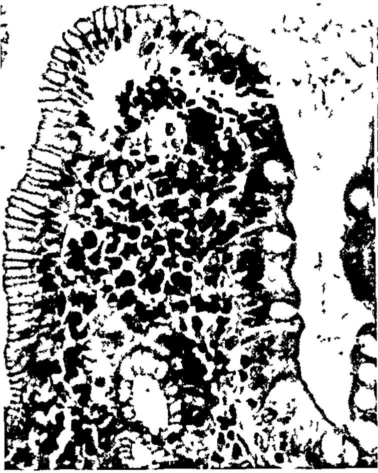

Fig 10 —Gastritis with severe tubular atrophy, showing the normal columnar cell gastric type surface epithelium changing over to intestinal epithelium with goblet cells and a striated border Hæmatoxylin and eosin ×400.

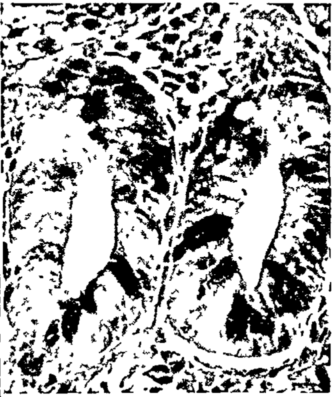

Fig 12 —Gastric atrophy in pernicious anæmia, showing Paneth cells filled with darkly staining granules situated at the bases of the intestinal type tubules Trichrome ×420.

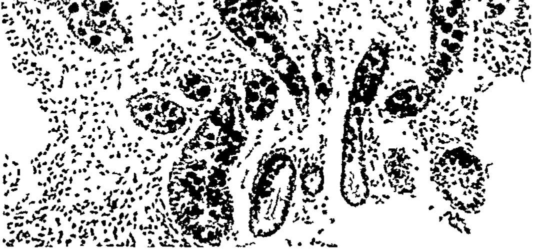

Fig 11 —Gastric atrophy in pernicious anæmia There is complete tubular atrophy and replacement by the intestinal type of glandular tubules The lamina propria is almost free of wandering cells Hæmatoxylin and mucicarmine ×140

never shows the denudation with actively regenerating flattened epithelium which is so commonly seen in gastritis. Where gastric-type epithelium does persist, it is always of high columnar type and stains intensely with mucin stains.

## DISCUSSION

The mucosal changes in gastritis and the mucosal atrophy of pernicious anæmia are probably diffuse over the whole extent of the body mucosa. In support of this assumption there was close correlation between the atrophy of chief and parietal cells observed in a given biopsy sample and the functional defect as observed in the histamine test meal (Wood *et al.*, 1949*b*). In addition the histological appearances of specimens from different sites are very similar. Therefore the small biopsy piece can reasonably be regarded as representative of the general state of the body mucosa.

Apart from alcohol, no known ætiological factor in the production of the gastritis was observed. A history of an immediately preceding alcoholic bout was obtained in three cases whose mucosa showed a superficial gastritis. On withdrawal of the alcohol healing was rapid. Biopsy was also performed upon twenty cases of chronic alcoholism with associated malnutrition. These patients all had enlarged fatty livers, which in some cases had progressed to portal cirrhosis. Several of them also showed peripheral neuritis. The biopsy specimens were obtained after two to three weeks' stay in hospital. In nineteen of these cases the gastric mucosa was normal or showed only minor deviations from normal. This finding is contrary to Faber's suggestion that minor grades of recurring damage to the gastric mucosa, as observed in those chronically addicted to alcohol, result in chronic atrophic gastritis. Nor would malnutrition appear to be an ætiological factor.

In two cases showing superficial gastritis, biopsy repeated a year later showed the same degree of change. There is thus no evidence of an inevitable progression of a superficial gastritis to a more atrophic gastritis. Rather the lesion is static, a particular degree of damage being produced by the unknown exciting agent.

The atrophy and intestinal metaplasia observed in the more severe gastritis could possibly be induced by the altered local conditions associated therewith. In pernicious anæmia these metaplastic changes are present to an advanced degree but there is no gastritis. Therefore one might postulate a general cause for the altered differentiation of the products of foveolar-cell multiplication in pernicious anæmia. The return to normal of the megaloblastic bone marrow with adequate liver therapy is not paralleled by a return of the gastric mucosa to normal. One-half of the cases investigated had received treatment for several years and yet showed no sign of a return of the mucosa to the gastric pattern. Some observers, on the basis of gastroscopic observations unsupported by histological examination, have claimed that true gastric regeneration occurs (Jones *et al.*, 1935).

## SUMMARY

1. The morphology of the mucosa of the body of the stomach in 150 patients suffering from a variety of dyspeptic symptoms and in whom an opaque meal showed no gross abnormality is described.

2. Changes, when present, could be grouped into two broad categories :—(*a*) superficial gastritis without atrophy of tubular epithelium, and (*b*) more deeply extending gastritis with atrophy of tubular epithelium.

3. Mucosa from patients with superficial gastritis due to excess of alcohol returned to normal after withdrawal of the alcohol.

4. In 43 cases of pernicious anæmia, complete or almost complete tubular atrophy combined with prominent intestinal metaplasia and absence of wandering-cell infiltration made up a pattern which is distinctive for this disease.

5. Adequate liver-extract therapy failed to alter these mucosal appearances.

I wish to express my gratitude to Dr Ian Wood, head of the Clinical Research Unit, and to Dr R. K. Doig, who secured the biopsy specimens. I also wish to thank Professor E. S. J. King for valued advice and criticism. The photomicrographs were prepared by Mr H. Mathhaei of the University of Melbourne.

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# ERYTHRÆMIC MYELOSIS IN A GIRL AGED 13 YEARS

John L. Emery

*From the Department of Pathology, Sheffield Children's Hospital*

(Plates LXIV and LXV)

Neoplastic change in the primitive red cells of the blood analogous to that of myeloid leukæmia is one to be expected, but such cases are extremely rare and very few of those described withstand critical examination. Quattrin (1940) accepted eight cases and Moeschlin (1947) considered that 12 cases, including one only in English literature (Israels, 1939), could be substantiated up to 1946.

The patient described below had abnormal nucleated red cells in her peripheral blood, up to a maximum of 550,000 per c.mm., for approximately two years. The course of her fatal illness resembled very closely that of the more common leukæmias.

## Case report

### *Clinical history*

Sylvia was born on 28 2 34, and apart from measles and pertussis had no illness in childhood. She was of purely English stock; her parents and two siblings are well and give normal blood counts. The only case of hæmic disease among her relations was that of her mother's sister, who died at the age of 26 with what was reported as pernicious anæmia.

Sylvia was first seen by us on 31.5 47, when she was 13 years old. She had been well until six weeks before admission, when she had become easily tired and her mother noticed that she was becoming progressively paler. Her gums later commenced bleeding when she cleaned her teeth.

No remarkable symptoms occurred until she was admitted to Worksop Hospital following a severe epistaxis, when an immediate blood transfusion was considered necessary. This transfusion did not completely stop the bleeding and she required a second transfusion five days later. Following the second transfusion she was transferred to the Children's Hospital, Sheffield.

**On admission**, 31.5.47, her general condition was good (weight 6 st. 4½ lb., height 5 ft. 1 in.) and the only abnormal physical sign found was a palpable spleen.

*Blood count.* Hb. 8 8 g./100 c c. R B C. 3·2 m /c mm.; W.B.C. 2800 per c.mm —neutrophils 1350, eosinophils 50, lymphocytes 1170, monocytes 50. Unidentified cells 180. Erythroblasts 1900 per c mm.

*Sternal marrow.* This showed a depression of the myeloid series and a relative increase in erythroblasts (table).

*General investigations.* Icteric index 9. Serum bilirubin 1·5 mg./100 c.c. Hijman's van den Bergh reaction produced a faint pink colour in 30 minutes. No occult blood detected in fæces. Urine normal. Coomb's test—direct and indirect—negative. Donath-Landsteiner and acid serum tests (Ham and Horack) negative. Schumm's test negative. Red cell fragility in saline normal. Reticulocyte count 2 per cent. (a very large number of nucleated red cells also contained reticulin). Bleeding and clotting times normal.

TABLE

*Three sample blood and sternal marrow counts, one in the early stages of the disease, the others* 11 *and* 14½ *months later, illustrating that the effect of a blood transfusion in lowering the peripheral erythroblast count was not reflected in a change in the marrow cytology*

| | 2.6.47 | 2.5.48 | 13.8.48 (After a transfusion) |
|---|---|---|---|
| **Peripheral Blood** | | | |
| Hæmoglobin (g. per 100 c.c.) | 8·8 | 5·5 | 5·6 |
| R.B.C. (m./c.mm.) | 3·2 | 1·9 | 2·4 |
| Nucleated cell count (per c.mm.) | 4500 | 550,000 | 75,000 |
| Leucocytes (per c.mm.) | 1700 | 3000 | 0 |
| **Marrow** | | | |
| Neutrophil polymorphs | 8 | 0·75 | 0·8 |
| " Stab " cells | 0 | 0·18 | 0 |
| Eosinophil polymorphs | 0 | 0·75 | 0 |
| Metamyelocytes | 7 | 0·94 | 0·4 |
| Myelocytes | 7 | 0·75 | 0·8 |
| Pre-myelocytes | 1 | 1·5 | 0·6 |
| Myeloblasts | >0·2 | 0·18 | >0·2 |
| Lymphocytes | 4 | 1·32 | 2·6 |
| Plasmocytes | 1 | 0·37 | >0·01 |
| Monocytes | >0·2 | 1·13 | >0·01 |
| Hæmocytoblasts | 1 | 1·77 | 0·4 |
| Pro-erythroblasts | 7 | 1·88 | 2·4 |
| Basophilic "normoblasts" | 22 | 7·0 | 5·0 |
| Polychromatic " normoblasts " | 15 | 28·1 | 31·9 |
| Orthochromic " normoblasts " | 25 | 38·1 | 28·9 |
| Karyorrhectic normoblasts | 0 | 14·7 | 25·6 |
| Mitotic cells | >0·2 | 1·13 | 0·2 |
| Megakaryocytes | >0·2 | 0 | 0·4 |

After transfusion of 1½ pints of whole blood, her general condition appeared excellent, her peripheral blood containing Hb. 9·5 g./100 c.c. and R.B.C. 3·3 m./c.mm., and she was discharged on 8.7.47.

**Readmitted** 23.7.47. Hb. 6·8 g./100 c.c. R.B.C. 2·4 m./c.mm., peripheral erythroblasts 2500 per c.mm. Two pints of whole blood were given.

*Effects of folic acid.* As an outpatient she was given folic acid in large doses and weekly marrow punctures were performed. No alteration was found in the marrow as a result of this medication, nor was there any apparent effect on the development of the anæmia.

**Readmitted** 1.9.47 to 3.9.47, when she received two pints of packed cells.

**Readmitted** 15.10.47, with severe bruising and epistaxis, her general condition, however, being fairly good. At this time she required several transfusions and her spleen was observed to be larger than previously.

*Blood count.* Hb. 5·2 g./100 c.c. R.B.C. 1·8 m./c.mm.; *W.B.C.* 3200 per c.mm.—neutrophils 1050, metamyelocytes 150, lymphocytes 2000, monocytes less than 50; erythroblasts 6300 per c.mm., consisting of primitive cells 50,

# ERYTHRÆMIC MYELOSIS IN A YOUNG GIRL

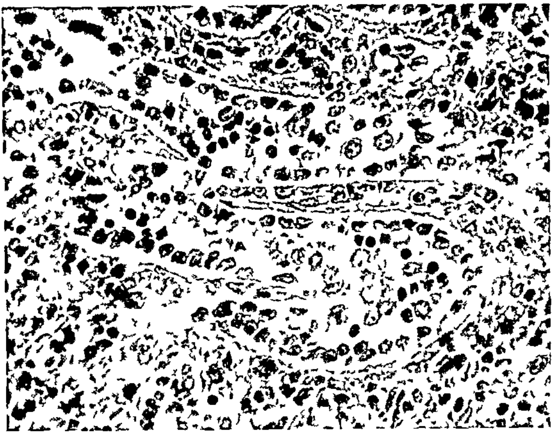

FIG 1 —Section of spleen showing well defined sinusoids with abnormal endothelium and erythroblasts in the lumen Hæmatoxylin and eosin ×440

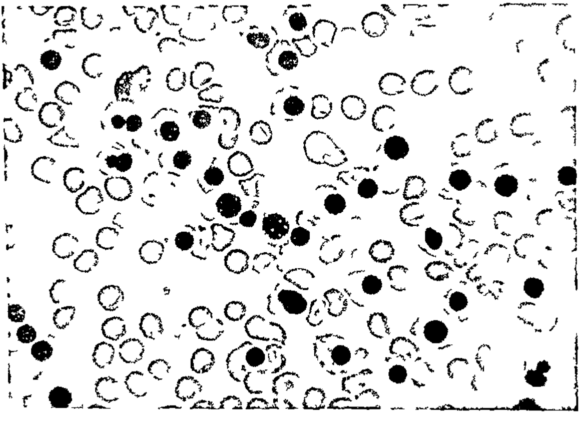

FIG 2 —Film of peripheral blood The transfused erythrocytes are easily distinguished from the patient's cells and a large number of erythroblasts are present Giemsa ×600

karyorrhectic cells 1000, early erythroblasts 360, polychromatic cells 2360, orthochromatic erythroblasts 2530.

*Other investigations.* Total serum proteins 7·9 g./100 c.c. (serum albumin 6·0 g., serum globulin 1·9 g.). Schumm's test negative. Fæcal bilirubin 490 mg. in 24 hours. W.R. negative. The blood was retested for hæmolysins and agglutinins with negative results. Slight rouleau formation occurred at 4° C. Gastric test-meal normal. Saline red-cell fragility normal. An attempt was made to estimate the survival times of the transfused erythrocytes, but owing to excessive rouleau formation no reliable results could be obtained.

The spleen had been enlarging during the course of the disease and was the only abnormal clinical physical finding. Since no other form of treatment had affected the course of her illness it was decided to remove her spleen.

*Splenectomy* performed, 21.11.47. The spleen was grossly enlarged, weighing 240 g. (normal weight for this age 90-110 g.), the surface being slightly granular, with many small vascular adhesions. A small spleniculus was also removed (fig. 1). The liver appeared enlarged but otherwise normal. The gall bladder and pancreas appeared normal and no enlarged glands could be found in the mesentery.

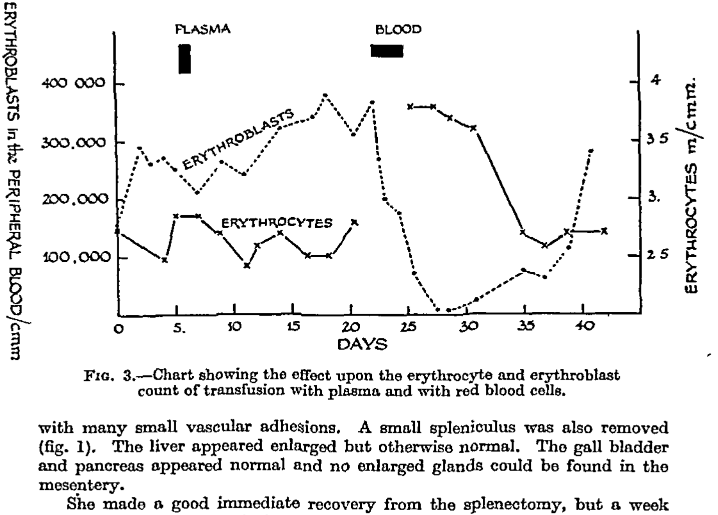

FIG. 3.—Chart showing the effect upon the erythrocyte and erythroblast count of transfusion with plasma and with red blood cells.

She made a good immediate recovery from the splenectomy, but a week later she showed signs of pleural effusion and developed an empyema which resolved without open drainage. She was discharged on 16.12.47.

**Readmitted 26.1.48.**

*Blood count (fig. 2). Hb. 7·5 g./100 c.c. Peripheral erythroblasts 350,000* per c.mm.; leucocytes 6000—neutrophils 3300, lymphocytes 2640; platelets 60,000 per c.mm.

*The effect of transfusing plasma and blood cells.* Following blood transfusion, the number of erythroblasts in the peripheral blood diminished very swiftly, but no coincident change could be found in the bone marrow (table, p. 396). This fall in peripheral erythroblasts could have been due to the presence of some maturation factor in the transfused blood. In order to exclude to some extent this possibility, the patient was given fresh plasma before one transfusion, the volume of plasma being equivalent to that of the blood cells to be given later. The plasma produced no change in the circulating cells in any way comparable to that of the packed cells given later (fig. 3).

*Lymph nodes.* There is no disorganisation of architecture. The nodes, and in particular the germinal centres, which at a casual glance appear to be absent, are almost acellular. This greatly reduced *cellularity affects all the lymphoid tissue of the body, including* the Peyer's patches and the thymus. The sinusoids and pulp of the lymph nodes contain pale-staining cells and erythroblasts but not in greater numbers than are present in sections of blood clot. Unlike the other organs, none of the lymph glands shows erythroblastic infiltration.

*Lung.* Many alveoli are filled with erythroblasts, and there is erythroblastic infiltration around some of the bronchioles.

*Liver* contains much iron pigment and there is diffuse infiltration of the portal tracts with erythroblasts.

*Pancreas, kidney, diaphragm, skin, intestinal mucosa and adrenals* all contain areas of erythroblastic infiltration (fig. 6).

*Left axillary region and related muscles.* These show destruction of tissue and large masses of bacteria, but with no recognisable inflammatory reaction.

*Brain, thyroid, pituitary* show no significant abnormality.

## DISCUSSION

The literature of erythræmic myelosis has been reviewed by Moeschlin (1940), di Guglielmo (1946), Mallarme and Moulonguet (1947) and Leitner (1949, pp. 166-173). The diagnostic criteria put forward by di Guglielmo and accepted by Britton and Neumark (Leitner, 1949) for the acute form of the disease are as follows:— (1) Severe anæmia from the start of the illness. (2) Irregular and usually remitting fever. (3) Splenomegaly. (4) Slight hepatomegaly. (5) An acute course of one to two months and invariably fatal. (6) Hæmic erythroblasts, with many atypical and immature forms. (7) Hyperplasia of the erythron with maturation arrest. (8) Proliferation of the reticulo-endothelial system.

Each of these points may be considered in turn in relation to the present case.

1. Our patient was admitted to hospital requiring an immediate blood transfusion. There was a history of progressive pallor for six weeks.

2. The temperature was very variable. For a few weeks following a transfusion it would remain normal, but as her transfused cells disappeared and she became more anæmic, pyrexial bouts occurred especially in the later stages of her illness, when there was an increasing tendency to sepsis. While in hospital she had occasional rigors.

3. Splenomegaly was present from the time that she was first examined.

4. The liver became enlarged later.

5. Without transfusions the patient would probably have died

within two months of the onset of symptoms. Life was maintained by means of blood transfusions for two years. Examination of blood films suggested that virtually all of the non-nucleated cells in the circulating blood were the transfused red cells. The few large non-nucleated cells seen in the films were numerically no greater than the free nuclei (fig. 2).

6. Up to 550,000 erythroblasts per c.mm. were present on occasion in the peripheral blood. This is a greater number than any hitherto reported in cases of this kind, the highest being 258,000 per c.mm. (Lattes, 1932). The cells were larger than those of the megaloblastic anæmias, the average diameter being 11 μ (fig. 4). They were mononuclear and did not resemble the giant erythroblasts described in leukemia as cells measuring more than 18 μ. These cells, described by Schwarz (1946), are taken to be cells in which the final stage of mitosis is defective. Most of the cells in the present case contained only one nucleus, although multinucleated cells were seen by Dr Lajtha in the peripheral blood culture. Maturation arrest was seen in the cultures, and would appear to be an inherent property of the cells and not due to any extracellular deficiency factor.

As the autopsy followed a prolonged state of sepsis and inanition and was not done until 50 hours after death, the cytology exhibited in the spleen biopsy probably represents the reticulo-endothelial system most characteristically (fig. 1). The cells lining the sinusoids of the spleen are large but do not appear to be proliferating or to be ingesting cells. These cells are abnormal, but it does not seem possible from their appearance to tell into which of the elements of the blood they were progressing. A similar difficulty arises in relation to many of the primitive cells found in the marrow smears and illustrated in fig. 5. Transitional forms from these cells to the hæmoglobinised cells were present. They do not appear to be either myeloblasts or normal reticuloendothelial cells. They are probably abnormal reticulo-endothelial cells corresponding to the cells in the sinuses of the spleen and an attempt to label them further would not seem to be helpful. Nowhere in the autopsy tissues did the lymph nodes show structural derangement. Infiltration by the abnormal blood-cells was present in the liver, pancreas and kidneys, and the bone marrow from the various sites examined showed uniform neoplastic changes.

The leucocytes of the blood were reduced in number throughout the whole course of the disease, but primitive forms were in approximately the normal ratio. The platelets were decreased in association with the hæmorrhagic crises. The predominant cells appeared to be more mature than those in the other two cases reported in this country, those of Israëls (1939) and Neumark (Leitner, 1949, p. 167). The marrow count resembled that of the case described by Nabholz (1942).

To which type of erythræmic myelosis is this case to be assigned? If the child had died at the time of the first emergency transfusion, a consistent leuco-erythroblastic picture. The polymorphonuclear count showed no shift to the right. There was marked thrombocytopenia throughout. Except on the last two occasions there was no macrocytosis, and the normal mean corpuscular hæmoglobin concentration excluded iron deficiency. The diagnoses first considered were secondary carcinomatosis of the marrow with a hidden primary and aleukæmic myeloid leukæmia. Neither of these possibilities could be substantiated either during life or at autopsy.

TABLE I

*Hæmatological findings*

| | 19.1.50 | | 20.1.50 | | 23.1.50 | | 26.1.50 | | 30.1.50 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Hæmoglobin (g./100 ml.) | 7·8 | | 7·5 | | 6·7 | | 6·0 | | 7·8 | |
| Red cells (M/c.mm.) | 2·73 | | 2·56 | | 2·44 | | 1·92 | | 1·90 | |
| Hæmatocrit (per cent.) | 24·1 | | 23·3 | | 20·8 | | 19·0 | | 23·6 | |
| Mean corpuscular Hb. (γγ) | 29 | | 29 | | 28 | | 31 | | 41 | |
| Mean corpuscular volume (c.μ.) | 88 | | 91 | | 85 | | 99 | | 124 | |
| Mean corpuscular hæmoglobin concentration | 32·4 | | 32·2 | | 32·2 | | 31·6 | | 33·0 | |
| Reticulocytes (per cent.) | 3 | | 2·6 | | 1·9 | | 1·2 | | 3·0 | |
| Leucocytes (per c.mm.) | 3600 | | 4100 | | 3600 | | 2700 | | 4700 | |
| | Per cent. | Total | Per cent. | Total | Per cent. | Total | Per cent. | Total | Per cent. | Total |
| Neutrophils— | | | | | | | | | | |
| Juvenile | 3 | 110 | 4·5 | 180 | 2·6 | 90 | 5 | 140 | 2 | 90 |
| Stab | 31 | 1120 | 33 | 1350 | 33 | 1190 | 38 | 1030 | 45 | 2110 |
| Segmented | 14 | 500 | 8 | 330 | 10 | 360 | 19 | 510 | 13 | 710 |
| Eosinophils | 1·5 | 50 | 0·5 | 20 | 0 | 0 | 0 | 0 | 0 | 0 |
| Basophils | 0·5 | 20 | 1 | 40 | 0·5 | 20 | 0·5 | 10 | 0 | 0 |
| Lymphocytes | 34 | 1220 | 32 | 1310 | 35 | 1260 | 18 | 490 | 24 | 1130 |
| Monocytes | 1·5 | 50 | 0·5 | 20 | 2 | 70 | 0 | 0 | 1 | 50 |
| Metamyelocytes | 2 | 70 | 5 | 210 | 3 | 110 | 4 | 110 | 5 | 240 |
| Myelocytes | 6 | 220 | 6 | 250 | 5 | 180 | 6 | 160 | 4 | 190 |
| Promyelocytes | 4 | 140 | 7 | 290 | 7 | 250 | 8 | 220 | 3·5 | 160 |
| Myeloblasts | 2·5 | 90 | 2·5 | 100 | 2 | 70 | 1·5 | 40 | 2·5 | 120 |
| Normoblasts— | | | | | | | | | | |
| Late | 500 | | 570 | | 540 | | 220 | | 1410 | |
| Intermediate | 100 | | 60 | | 90 | | 10 | | 380 | |
| Early | 0 | | 0 | | 20 | | 0 | | 0 | |
| Platelets | 30,000 | | 35,000 | | 2000 | | 2000 | | 4000 | |

Table II displays the differential count, made on 1000 cells, of a sternal-marrow specimen aspirated in the usual manner with a Salah needle from the manubrium sterni on 20th January 1950. The smears were made direct and without the use of any anticoagulant. The marrow was highly cellular and the erythropoietic series predominated. The most remarkable feature was the presence of some 25 per cent. of erythroblasts with from 10 to 100 nuclear fragments, usually

## ABNORMAL ERYTHROBLASTIC KARYOKINESIS

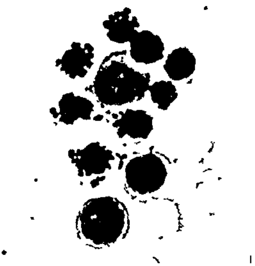

FIG 1.—A group of erythroblasts with scattered chromosomes or chromosome fragments.

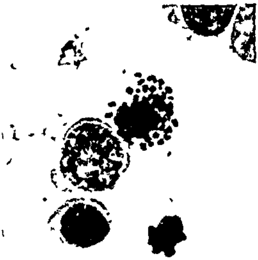

FIG. 2—Numerous chromosome particles in an erythroblast

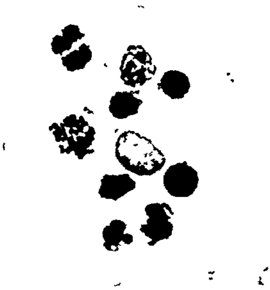

FIG. 3—Several mitotic abnormalities in a group of erythroblasts

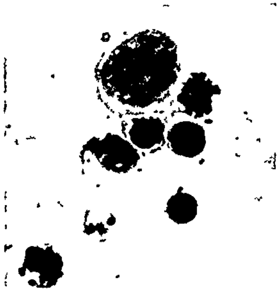

FIG 4—A large primitive "blast" cell with several nucleoli

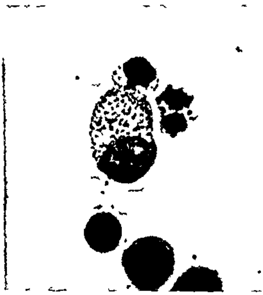

FIG 5—One of the "unidentified cells" of this marrow, showing the large azurophil cytoplasmic bodies

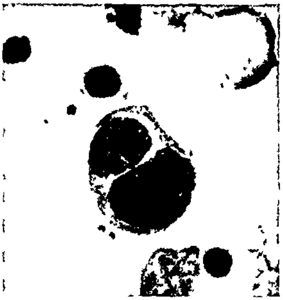

FIG. 6—An early giant erythroblast with unequal nuclei

FIGS. 1-6.—From the unusual marrow reported in this paper.

ABNORMAL ERYTHROBLASTIC KARYOKINESIS

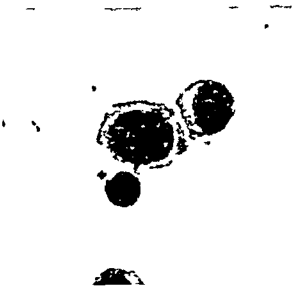

FIG 7 —Unequal daughter erythroblasts resulting from an asymmetrical mitosis

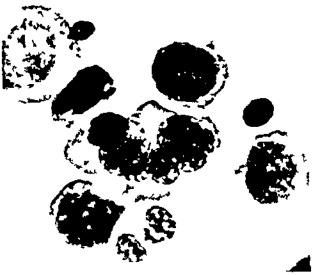

FIG 8 —Four unequal nuclei in a giant erythroblast resulting from asymmetrical multipolar mitosis without cytokinesis

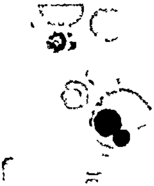

FIG 9 —Two unequal nuclei in a late erythroblast

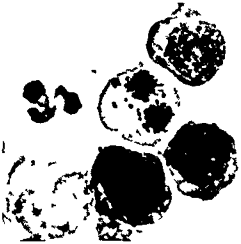

FIG 10 —Erythroblast in anaphase with several chromosomal masses detached from the spindle

FIG 11 —A similar erythroblast with 2 reconstituted nuclei and several accessory nuclear masses

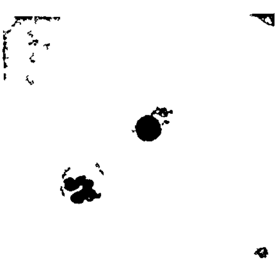

FIG 12 —Two erythroblasts the lower showing rosette formation the upper accessory nuclear material

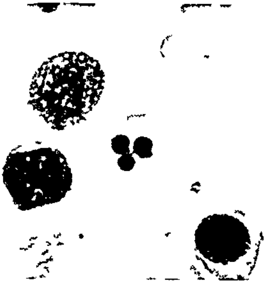

FIG 13 —A late erythroblast with nucleus showing rosette appearance

FIGS 7–13 —From an active erythropoietic marrow in a case of nutritional macrocytic anæmia of pregnancy

round or oval, but occasionally elongated and resembling chromosomes (figs. 2 and 3). The fragments were Feulgen-positive. There were 2·2 per cent. binucleated erythroblasts, and occasional giant normoblasts with a diameter of 15-25 μ but no multipolar mitoses were observed. The primitive " blast " cells included in the count were large—20-30 μ in diameter—with nucleus 15-25 μ containing 3 or 4 pale lilac nucleoli. The nuclear chromatin was very finely stippled or in the form of a fine network of interlacing strands. The cytoplasm was moderately basophilic and sometimes showed differentiation into spongioplasm and hyaloplasm, and occasionally pseudopodia were formed. The unidentified cells had a rather similar nuclear structure, but more cytoplasm, in which large azurophil rods or granules were present.

TABLE II

*Sternal-marrow count, 20th January* 1950

| | Neutrophil | Eosinophil | | Late | Intermediate | Early |
|---|---|---|---|---|---|---|
| Polymorphs . | 0·7 | 0 1 | Normoblasts | 21 8 | 6 9 | 13 1 |
| Metamyelocytes . | 2 7 | 0 5 | Binucleated normoblasts | 1 0 | 0 1 | 1 1 |
| Myelocytes . | 5 5 | 0 4 | | | | |
| Promyelocytes | 1·7 | | Pro erythroblasts | 2 8 | | |
| " Blast " cells . | 1 9 | | Normoblasts with nuclear fragmentation and scatter | 25 7 | | |
| Reticulum cells . | 2 7 | | | | | |
| Unidentified cells | 0 8 | | | | | |
| Monocytes . | 0 1 | | Normal mitotic figures, 4 2 | | | |
| Lymphocytes | 6 0 | | Myeloid erythroid ratio, 1 : 3 3 | | | |
| Plasma cells . | 0 2 | | Total cell count, 150,000/c mm. | | | |
| | | | Megakaryocytes <1 per 10,000 nucleated cells | | | |

All figures are percentages unless otherwise stated.

All these unusual types of cell are illustrated in figs. 1-6.

## *Post-mortem examination*

This revealed bilateral bronchopneumonia and numerous small subdural and subarachnoid hæmorrhages. The cardiovascular, urogenital, alimentary and endocrine systems showed no gross abnormality and no evidence of neoplasm was found at any site. The spleen was slightly enlarged (282 g.), with a soft purplish cut surface. It contained several firm, well-defined, dark red hæmorrhagic infarcts beneath the capsule, and areas of extramedullary hæmopoiesis outside the capsule. The liver showed macroscopically a mottled cut surface, and microscopy revealed large-droplet fatty change in the centres of the lobules and excessive brown-atrophy pigmentation.

Deep red, jelly-like, soft marrow extended throughout the shaft of the femur, into the head and to within 4 cm. of the distal end. Similar red marrow was present in sternum and vertebræ. Microscopical examination of the marrow showed rich cellularity, with increase especially of nucleated red cells. Many of these, particularly the late normoblasts, showed the same fragmentation of nuclei as had been observed in the marrow smears made during life.

## *Discussion*

Disturbances of mitosis in human erythropoiesis, with cellular gigantism, multinucleated erythroblasts and multipolar mitotic figures, have been described in both megaloblastic and normoblastic marrows. Limarzi and Levinson (1943) recorded a case of "giant erythroblastosis" in which a non-megaloblastic marrow showed primitive giant erythroblasts, multipolar mitoses and complicated amitotic division of the nucleus. Schleicher (1944) described two cases with similar atypical erythroblasts, which he believed to be produced by defunct reticulum.

Schwarz (1946) reviewed the subject of cellular gigantism and pluripolar mitosis, and described the disturbances that may occur in karyokinesis of the erythroblast. Apart from gigantism and multipolar division, these may be grouped under three heads.

(*a*) Asymmetrical mitosis, usually only marked in multipolar mitosis, producing unequal daughter cells or, if cytokinesis does not occur, unequal nuclei. A modification of this process is seen when a few aberrant chromosomes become transformed into one or more small accessory nuclear masses, present in one of the daughter cells.

(*b*) Rosette formation, with bridges between chromatin masses, produced by a halt in metaphase with groups of fused chromosomes linked probably by interzonal spindle fibres.

(*c*) Interruption of karyokinetic movement, with agglomeration, scatter, or disintegration of the separated chromosomes. Such an interruption may produce a cell filled with scattered chromosomes or chromosome fragments.

The first two of these three mechanisms can be seen very commonly in pathological marrows, particularly where there is active normoblastic or megaloblastic hyperplasia. They are illustrated by figs. 7-13, taken from smears of sternal marrow from a case of nutritional macrocytic anæmia of pregnancy.

The type of interrupted mitosis resulting in breaking and scattering of chromosomes is, however, much less common. The involvement of over 30 per cent. of the nucleated red cells of the marrow in such a process of karyokinetic interruption and chromosomal fragmentation must, therefore, be considered an extreme rarity. We have never before seen anything approaching this picture.

The exact mechanism of production of the scatter and fragmentation which presumably involves a breakdown in polar forces, is not known.

## *Summary*

An old woman with a leuco-erythroblastic anæmia of unknown ætiology had an extreme normoblastic hyperplasia of the marrow. Over 30 per cent. of the nucleated red cells of the marrow showed fragmentation and scattering of the chromosomes.

This very rare abnormality is compared with other more common disturbances of erythroblastic karyokinesis.

We gratefully acknowledge the advice and encouragement of Sir Lionel Whitby and the assistance of Mr B. W. Gurner in preparing the photomicrographs. One of the authors (F. G. J. Hayhoe) is the holder of an Elmore Research Studentship.

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616 . 24—002 . 185 : 582 . 282 . 123 (*Aspergillus fumigatus*)

# A CASE OF PULMONARY ASPERGILLOSIS

C. F. Ross

*Ashford Hospital, Ashford, Middlesex*

(Plates LXVIII and LXIX)

Although there is an extensive literature on pulmonary aspergillosis in animals, mainly in birds, this disease remains an uncommon one in the human subject. It has not often been reported in this country, though two proved cases in coal-miners have recently been described by Heppleston and Gloyne (1949) and a probable case by Delikat (1945). The literature, however, does not give a true idea of the incidence of the disease, because many cases are never reported. Duncan (personal communication) states that he examined material from sixteen proved cases in the years 1946-1949 and only two of these were published. It would appear, therefore, that the disease is commoner and more important than has been supposed.

John Hughes Bennett (1844) was the first to recognise parasitic fungi growing in living animals when he described, in 1842, in addition to those occurring in lower animals, certain fungi growing in tuberculous cavities in man. Virchow (1856) coined the term "mycosis" for fungous infection and described the first cases of human pulmonary aspergillosis, though he was of the opinion that a case reported some years earlier by Sluyter (1847) as being one of Mucor infection was really due to Aspergillus. The literature on the subject increased greatly during the latter half of the nineteenth century and culminated in the classical monograph of Rénon (1897), who studied aspergillosis in all its aspects and gave a complete bibliography up to 1896. One of the early cases reported in this country was that of Boyce (1892-93).

## Mycology

The cause of pulmonary aspergillosis is *Aspergillus fumigatus* (Fresenius), a fungus of the genus Aspergillus Micheli, 1729. Although it is included in the class Ascomycetes, perithecia and ascospores have never been found with certainty in this fungus, and for that reason some authors have classified it in the group of Fungi imperfecti. Interested readers are referred to Thom and Church (1926), Thom and Raper (1945, p. 148), Wolf and Wolf (1947) and Lutz (1948) for further details.

Aspergilli are inhabitants of soil, especially when it contains an appreciable amount of organic material such as decaying vegetable matter (Thom and Raper, 1945, p. 150). They play a big part in decomposition processes and grow successfully at temperatures up to 49° C., when most other fungi are inactive, paving the way for bacterial action. The spores, which are very resistant, can be found in dust, dry leaves, chaff (Chortis, 1949), tea-dust (Castellani, quoted by Gohar, 1948) and imperfectly dried grain, especially rye. The fungi are able to withstand a temperature of —12° C. and are notorious laboratory contaminants.

*Aspergillus fumigatus*, which was first adequately described by Fresenius (1850), consists of a mycelium of hyphæ 2-3 μ in diameter. Here and there the cells become somewhat enlarged (5 μ diameter) to form foot cells which have thicker walls and give rise to long smooth-walled conidiophores 200-300 μ in length and 2-8 μ in diameter. These end in club-shaped expansions called vesicles, 20-30 μ in diameter, which bear over the distal half a single series of many crowded bottle-shaped phialides (sterigmata) 5-10 μ long and 2-4 μ at their maximum diameter (fig. 1).

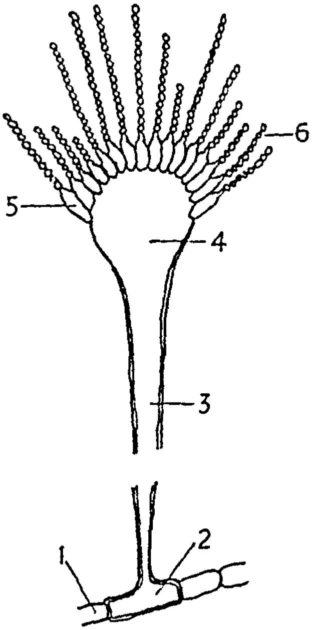


Fig. 1.—Diagrammatic sketch of *A. fumigatus* showing (1) hyphal cell, (2) foot cell, (3) conidiophore, (4) vesicle, (5) phialide (sterigma), (6) conidia (spores).

The fungus grows well on all the ordinary media, but Raulin's fluid (Raulin, 1869) or one of its many modifications is advised by several authors (Sartory and Bailly, 1923; Jacobson, 1932, p. 270). Rénon (p. 235) suggested the use of sputum-culture in slide cells as follows: sputum is spread on a microscope slide and covered with Raulin's fluid; another slide is placed on top, the edges are sealed with vaseline, and the whole incubated at 37° C., the optimum temperature for this fungus. The synthetic medium inhibits the common bacteria and after a few passages the fungus can be obtained in pure culture. The appearances do not vary very much on different media, though the colour is said to be brownish on alkaline media (Sartory and Bailly), while on acid media it is greenish. After 24 hours a tufted aerial mycelium is produced which may be velvety or floccose. Since the vegetative mycelium is white, the colour of the colony is also white until the conidia are developed, which may be for 24 hours, or, in some strains, 2-3 days. The subsequent colour changes depend on the composition and reaction of the medium. On media of relatively low *p*H, high carbon and low nitrogen content the mycelial bed is white, thin and papyraceous while the aerial growth is short, thin and of a bluish-green colour, changing with age and the rising *p*H to grey or greyish-brown. On media rich in nitrogen, such as peptone-agar, and low in carbon, the colour of the colony is less bluish and more definitely green, often olive green, and in this case the colour change to brownish comes earlier and the ultimate colour may be deep brown or even blackish-brown (Duncan, personal communication). Densely crowded conidiophores are found with the characteristics previously described.

*A. fumigatus* produces the powerful but highly toxic antibiotics "fumigatin" and "fumigacin", which are active against Gram-positive bacteria. In this connection it is interesting to note that nearly forty years ago it was reported that filtrates of cultures of the fungus attenuated human tubercle bacilli (Vaudremer, 1913). This has been verified recently by Soltys (1944) and a partially purified substance active against tubercle bacilli in high dilution was recovered from filtrates by Asheshov and Strelitz (1945). They thought this was probably fumigacin.

## PATHOLOGY

The following short account is based on the descriptions of Rénon (1897, pp. 239-254 and 261-267), Macaigne and Nicaud (1926), Schneider (1930), Jacobson (1932, p. 274) and Chortis (1949).

Pulmonary aspergillosis occurs either as a primary disease or, more commonly, in association with other lung lesions (*i.e.* secondary).

*Primary infection.* Infection occurs via the bronchi and bronchioles, throughout which the fungus ramifies. Penetration of the tissues takes place and areas of necrosis result, with formation of abscesses and cavities, or consolidation of the lung. The process may spread slowly throughout the lung, but is more usually confined to one or other upper lobe, often towards the apex. Sometimes the condition is arrested, with fibrosis and calcification as the end result. When fibrosis is extensive, the patient may develop "cor pulmonale" and die from heart failure. Invasion of blood-vessel walls is common and thrombi may be formed which contain mycelium, *i.e.* there is a thrombosing pulmonary arteritis and phlebitis. Atheroma of the pulmonary arterial tree is often found, even while the patient is still quite young (Cawley, 1947).

Microscopically the appearances are those of a suppurative pneumonia with necrosis, and colonies of the fungus are seen in broken-down alveolar spaces and spreading through the walls of air-passages and blood-vessels. It is usual for mycelium alone to be present, but occasionally fruiting heads are found. This is possibly related to the fact that the mycelium can grow under relatively anaerobic conditions, but for the formation of sporing heads an adequate oxygen tension is required. Although from a study of the literature systemic aspergillosis appears to be rare, Duncan informs me that there was abundant evidence of hæmatogenous spread in the material studied by him. Cases have also been reported by

Cawley (1947), Gerstl, Weidman and Newmann (1948), and by Grekin, Cawley and Zheutlin (1950).

*Secondary infection.* In cases of chronic bronchitis, bronchiectasis, the pneumoconioses, tuberculous cavitation, infarction, bronchopneumonia and carcinoma, for example, the fungus may be encountered as a secondary invader. The first cases of this kind to be described were those of Virchow, and indeed most of the recorded cases of pulmonary aspergillosis have been secondary to chronic pulmonary disease.

### *Experimental pathology*

The most susceptible laboratory animal is the pigeon, but both the rabbit and guinea-pig may be used for experimental purposes. The dog and cat appear to be resistant. The intravenous route of inoculation is more certain than any other, but in pigeons intratracheal or intraperitoneal injection causes death in 10-20 days. Ingestion or subcutaneous injection has no effect.

When a suspension of the fungus is injected intravenously, the animal dies in from 2 to 7 days, depending on the number of spores in the inoculum. Tuberculoid lesions are found in many organs, including the lungs, liver, spleen, kidneys, muscles, intestine and bone-marrow. For some unknown reason the liver is mainly affected in pigeons and the kidneys in rabbits and guinea-pigs. Microscopically, the lesions are granulomata containing the mycelium. It may be mentioned here that when sections of these lesions are stained by Gram's method, the mycelium is usually Gram-negative but may be Gram-positive in patches.

## CLINICAL MANIFESTATIONS AND DIFFERENTIAL DIAGNOSIS

The onset may be acute, in the form of pneumonia, as in the cases of Delikat (1945), Gajardo and Honorato (1948), and Nordén (1948). More often the illness is a chronic one, and some cases go on for a very long time, like that reported by Macaigne and Nicaud (1926), whose patient had had symptoms for over fifty years. In these cases the commonest symptom is hæmoptysis, which may occur at intervals over long periods (Danielson, 1933), leading to a mistaken diagnosis of tuberculosis (Bergman and Henschen, 1930; Hobby, 1949; Chortis, 1949). This is the more likely in that the radiological changes in the lungs may be indistinguishable from that disease. Hertzog *et al.* (1949) reported a case which mimicked acute miliary tuberculosis and at autopsy showed lesions in the lungs resembling those seen in experimental aspergillosis.

All authors are agreed that one of the differential points in the diagnosis is the relatively much better general health of the patient with chronic aspergillosis than of a patients with pulmonary tuberculosis presenting similar X-ray appearances. The patient does not look so ill and there is less rapid loss of weight. Sputum may be

PULMONARY ASPERGILLOSIS

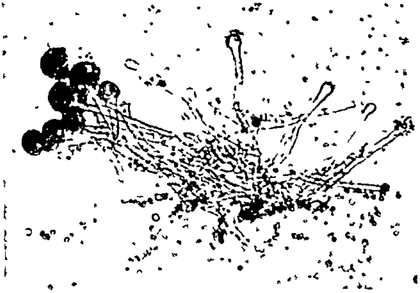


FIG 2 — Wet preparation of material removed from one of the lung cavities at autopsy, showing conidiophores and heads Unstained ×90.

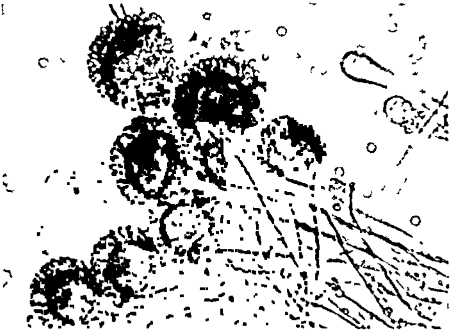


FIG 3 — Same as fig 2 but at higher power. Most of the conidia have become detached and are visible in the background ×400.

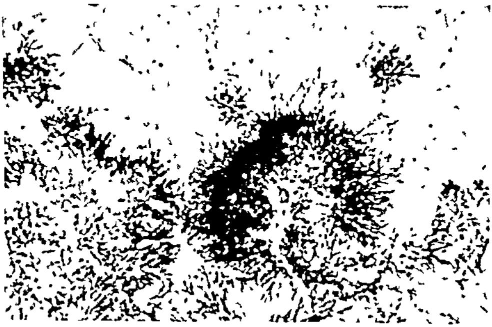


FIG. 4 — Section of lung taken from the edge of the lesion to show the advancing mycelium H. and E. ×90.

## PULMONARY ASPERGILLOSIS

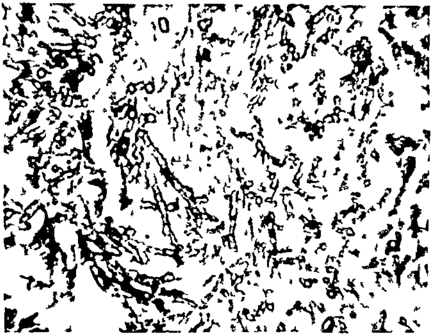
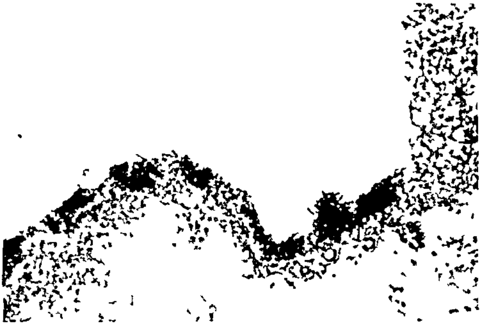


FIG 5 —Section of affected lung showing typical mycelium invading a blood vessel wall H and E ×400

FIG 6 —Section through a small cavity in the lung to show lining by a dense felt of mycelium, with a few conidiophores and fructifying heads in the right lower corner H and E ×90



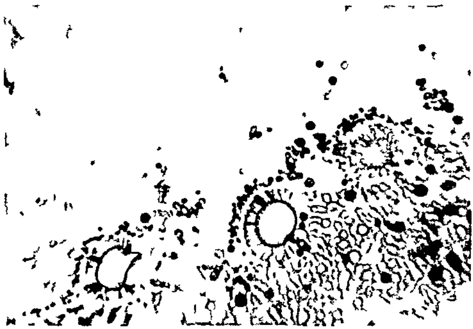


FIG 7 —Same as fig 6 but at higher power to show the fruiting heads ×400

abundant, but is consistently devoid of tubercle bacilli unless there is, as occasionally, coincidental or supervening tuberculosis. When this occurs, the disease is likely to be rapidly fatal. From time to time the fungus has been found in the nose and throat of normal individuals and may sometimes give rise to asymptomatic infection (Donaldson *et al.*, 1941-42).

Pulmonary aspergillosis is said to be an occupational disease, and a careful enquiry into the patient's occupational history and hobbies is worth while. In the State of Illinois it has been accepted as an industrial risk for workmen's compensation (Coe, 1945). Agricultural workers, fur cleaners and hair combers—who use rye flour for degreasing—and bird breeders, especially pigeon breeders, who feed the young fledglings with contaminated grain from their own mouths, as described by Rénon, or who contract the disease from infected birds during this process, are the main classes of person liable to infection. It is probable, however, that the fungus takes hold only when the resistance of the person is already lowered. If the disease depended on mere exposure to inhalation of large numbers of spores it would be a common disease, but, to quote Duncan again, not a single case was found in the occupational group of mouldy-dust inhalers. Sometimes there is no occupational cause to be found, as in the case reported by Nordén, where, after a thorough investigation, *A. fumigatus* was found in the air of the cellar of the patient's house.

### *Diagnosis*

This may be very difficult, because of the appearance of the fungus as a secondary invader in other lung diseases. However, the main disease from which primary pulmonary aspergillosis has to be distinguished is tuberculosis. The diagnosis rests on the absence of tubercle bacilli and the demonstration of *A. fumigatus* in the sputum. This may be determined by direct microscopy, especially if attention is directed to any minute greyish or yellowish flecks which may be present. Final proof requires, of course, culture of the fungus, when the characteristic appearances described above will be seen. An intradermal test, said to be specific, has been described by Macaigne and Nicaud (1927). As in all diseases, the laboratory results must be interpreted in the light of the clinical and other findings.

It seems not unlikely that this disease, under the guise of chronic tuberculosis, is more frequent than is commonly supposed. Attention may be drawn to the warning given by Wahl *et al.* (1928) that small mycelial fragments may retain the stain in the Ziehl-Neelsen method and be mistaken by the inexperienced for tubercle bacilli.

## TREATMENT AND PROGNOSIS

The only treatment of any value is the exhibition of iodides to the limit of tolerance. Sulphonamides are, perhaps, useful in combating bacterial infection of the diseased lung (Schneider). Sulphonamides and penicillin were used by Nordén to treat a case, but the patient developed a lung abscess and recovered only after iodides were given. Sulphadiazine, penicillin, streptomycin and aureomycin were all without effect in the case reported by Grekin *et al.*

The prognosis in chronic cases is fairly good, since the disease progresses slowly, giving time for full investigation and the discovery of the fungus, and the subsequent opportunity for treatment with iodides, to which the condition readily responds. In acute cases, if the disease is not recognised and treated early, the outcome is likely to be fatal.

## Case report

The patient was a man, 50 years of age, who died after total gastrectomy for generalised carcinoma of the stomach. He had had a partial gastrectomy three years previously for a gastric ulcer which was reported as showing no evidence of malignancy. He was a general labourer and there was no occupational history as regards his lung condition.

### *Post-mortem examination*

*Thorax.* There was no free fluid in the pleural cavities. The right lung was adherent at the apex. In the right upper lobe there was an area of consolidation occupying almost two-thirds of the lung substance and sharply demarcated from the rest of the comparatively normal lung. The overlying pleura was thickened and vascular. On cutting the mass, there was moderate resistance to the knife and the cut surface presented the following appearance. The lung substance was largely replaced by numerous areas of a rust-red colour in which were some patches of anthracosis. Many of these areas were surrounded by a narrow zone of firm white tissue 0·5-1 mm. thick, outside which was a thin red line of hyperæmia and some of them showed central cavitation. These cavities were from 0·5 to 1 cm. diameter and were filled with a fine grey dust which rose up like smoke when the affected portion of lung was cut through. Other cavities contained scattered greyish granules, 0·5 mm. in diameter, which could easily be removed on the point of a needle. Some cavities contained a number of these granules. The rest of the right lung showed only congestion and the left lung was normal apart from some congestion at the base. There was no evidence of similar lesions in any of the other viscera.

Direct microscopical examination of the material from the affected lung showed masses of fungal mycelium and fruiting heads (figs. 2 and 3) and a provisional diagnosis of *Aspergillus fumigatus* was made. Cultures on Sabouraud's medium were sent to Dr J. T. Duncan who confirmed the diagnosis.

### *Histology*

Sections of the lung showed a suppurative pneumonia, with areas of necrosis and cavitation, cut off from surrounding lung by a zone of granulation tissue. Lining and projecting into the cavities and permeating the adjoining tissues were masses of fungal mycelium, which was also present in bronchioles and was penetrating the pulmonary vessels. Some arteries and veins were thrombosed, the fungal mycelium forming a continuous network through vessel walls into the clot. In some places fruiting heads were also present. Elsewhere masses of spores were seen, some of which appeared to be germinating (figs. 4-7). These findings are similar to those described in cases of primary pulmonary aspergillosis.

## Summary

A short general account, clinical and pathological, of pulmonary aspergillosis is given, together with a report of a case of the primary type discovered incidentally at autopsy in a man of 50.

My thanks are due to Dr H. G. Broadbridge (H.M. Coroner for West Middlesex) for permission to report this case and to Mr E. A. Targett for help with the photography. I am deeply indebted to Dr J. T. Duncan for his report on the fungus and for helpful criticism and advice.

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# AN UNUSUAL RECURRING TUMOUR FORMED OF CONNECTIVE TISSUES OF EMBRYONIC TYPE (SO-CALLED MESENCHYMOMA)

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(Plates LXX-LXXIII)

Gilmour (1943) described a peculiar, histologically benign, recurring tumour consisting of connective tissues which he considered to be embryonic; he suggested that it could be called a mesenchymoma. The patient died four years after Gilmour's paper appeared, and her case is reviewed (case 3) in the present paper, in which three new cases of the same type of tumour are also recorded.

## Case reports

A macroscopic description of the specimens is given, where available, after the case-history of each patient. Because of their similarity, the histological findings in the four cases are dealt with together subsequently.

### Case 1

*Clinical history*

H. B., male, born in 1912, was admitted to the Wingfield-Morris Orthopædic Hospital in April 1948 with a smooth, tense, painless mass involving the lower half of the right thigh posteriorly. It had been present and enlarging for 18 months. There was no other abnormality. The clinical diagnosis was large semimembranosus bursa or sciatic neurilemmoma. At the first operation (17th April 1948), a pinkish-yellow, encapsulated tumour protruded from between the medial and lateral hamstring muscles when the deep fascia was incised; the sciatic nerve was tightly stretched over the tumour, but was easily dissected free. The tumour was removed intact after ligation of a few large vessels at its sides. By the 12th May 1949 a smooth, painless mass had formed at the site of the original tumour. In September 1949 the tumour was larger and amputation below the lesser trochanter was performed. In January 1951 the patient was in excellent health.

*Morbid anatomy*

The original tumour was solid and fairly soft. It weighed 802 g. and measured 19 × 11 × 9·5 cm., its surface was studded with low, rounded protuberances and there was a thin, intact, smooth capsule.

When bisected (fig. 1), the tumour resembled a neurilemmoma. Broad septa divided it into lobules from the sunken cut surface of which mucoid fluid exuded. The larger lobules were partly opaque and white or yellow, partly translucent and grey; the smaller lobules were translucent and grey or yellowish. The amputation specimen showed a tumour, measuring 17×13×7 cm. and weighing 676 g., deep to the hamstring muscles; its appearance was similar to that of the original specimen. The thin capsule was easily separable from the surrounding tissues except at the lower pole, where extensive scarring involved the bifurcation of the sciatic nerve, which ran deep to the tumour. Four rounded nodules, each 2×1·5×1 cm., were adherent to the capsule. Three rather smaller nodules were present beside the popliteal vessels at the lower pole of the tumour and a fourth was situated in the intermuscular fascia 3 cm. above the upper pole.

## Case 2

### *Clinical history*

E. H., female, born in 1906, was admitted to the Worcester Royal Infirmary in August 1948 with three years' history of a slowly enlarging tumour in the medial part of the lower third of the right thigh. There was no other abnormality. Radiography showed a normal femur. At the first operation (11th August 1948), an encapsulated, lobulated, gelatinous tumour, weighing 700 g. and measuring 17×12×10 cm., was found deep to the vastus medialis and extended back round the femur. Removal of the tumour was difficult owing to bleeding. By October 1949 the tumour had recurred and now extended well up the thigh. Radiography showed a periosteal reaction at the medial side of the lower third of the femur. The radiograph of the chest was normal. On the 31st December 1949 amputation through the upper third of the femur was performed. In January 1951 there was no sign of recurrence or metastases.

### *Morbid anatomy*

The amputation specimen showed an encapsulated tumour, 28×12×8 cm., in the lower half of the antero-medial aspect of the thigh, displacing and compressing the muscles and extending far along the intermuscular fasciæ. The tumour, which was similar to that in case 1, was easily enucleated, except where its capsule was adherent to the scar of the previous operation and to the periosteum over a small area on the medial side of the lower third of the femoral shaft. Here there was periosteal thickening, with a little bony overgrowth.

## Case 3

### *Clinical history*

M. C., female, born in 1900. Gilmour records that this patient first noticed the tumour in 1923 as an egg-sized lump in the centre of the lower part of her back. As far as we can learn, the original tumour was excised within a year of its appearance and the first recurrence, noticed 4 or 5 years later, was treated

MESENCHYMOMA

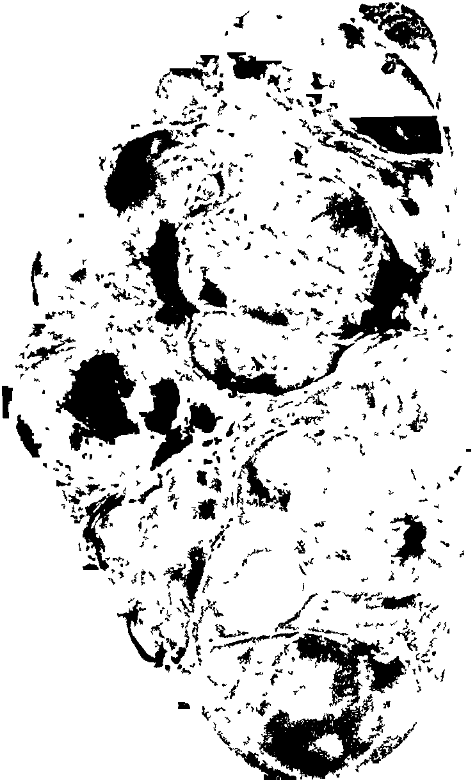


FIG 1 —Case 1 Cut surface of original tumour after bisection ×c 0 8

by excision followed by a few doses of X-rays. Further recurrent tumours were excised in 1933, 1935, 1938, 1939 (twice) and 1940 (twice). In January 1943 she was admitted to the Essex County Hospital, Colchester, with recurrence in the upper lumbar region. This tumour consisted of two lobes, each about 12·5 cm. across and adherent to the skin. The lower mass was excised on the 16th January 1943, with difficulty owing to infiltration between the fasciculi of the left erector spinæ. On the 20th February 1943 the upper lobe was removed, with gelatinous extensions into the right erector spinæ. Gilmour's description of the tumour corresponds with that in case 1.

*Progress.* The patient was referred to St. Thomas's Hospital, London, for radiotherapy. In May 1943 a dosage of 1800 r. was given to the loins over 12 days. In October 1943 the first recurrence since the operation in February 1943 appeared in the lower part of the irradiated area. A dosage of 2400 r. was given over 20 days, and in January 1944 only a small plaque remained palpable. Further recurrences at progressively shorter intervals necessitated 14 courses of deep X-ray therapy between June 1944 and February 1947, the dosage ranging from 1000 to 2100 r. over periods of 12 to 25 days. After each course the tumours regressed temporarily, with early recurrence within or outside the irradiated areas, and eventual ulceration. No evidence of metastasis was found on repeated clinical and radiographic examination between January 1943 and July 1947. By July 1947 further radiotherapy was out of the question because of damage to the skin. In August 1947 a course of three injections of lead phosphate was started. Five weeks later the tumours of the back were somewhat smaller, but the patient's general condition was worse and a mass "as large as her head" was found in the left iliac fossa and loin. A second course of lead-phosphate injections was given. The patient died of exhaustion on the 3rd October 1947. There was no necropsy.

## Case 4

### *Clinical history*

T. W., male, born in 1899, was admitted to the Queen Elizabeth Hospital, Birmingham, in September 1946 with a firm, slightly painful swelling in the left buttock of 8 weeks' duration. When lying on the affected side pain radiated down the back of the leg. At the first operation (19th September 1946) a large, encapsulated, lobulated tumour was removed by diathermy from deep to the gluteus maximus. Deep X-ray therapy, 3000 r. over 28 days, was given in November 1946. Recurrence was found in March 1948. At the second operation (12th March 1948) an encapsulated tumour, 8 cm. in diameter, was removed from deep to the gluteus maximus, where it was in contact below with the sciatic nerve, which was not involved. By July 1949, persistent aching in the buttock and occasional cramps in the left calf had been present for a year; three firm, tender masses were present in the left buttock along the line of the sciatic nerve; there was partial anæsthesia of the back of the thigh. At the third operation (22nd July 1949) a soft tumour beneath the glutei, encapsulated externally but invading the muscles of the gluteal region and of the back of the thigh, was resected together with invaded muscle and a 5 cm. length of involved sciatic nerve; it was thought probable that small, inaccessible nodules escaped observation. On the 16th August 1949 a hind-quarter amputation was performed in view of the probability of further recurrence with the risk of inward extension through the sciatic foramina; no tumour was seen during the operation. In August 1950 there was a large mass in the left side of the abdomen, rising out of the pelvis and obstructing the left ureter; chest radiographs were normal and the general condition was good. The tumour was inoperable and a biopsy was taken. The patient died at home a few weeks later. There was no necropsy.

### *Morbid anatomy*

(*a*) The specimen from the third operation weighed 646 g. and comprised incompletely encapsulated, flattened, ovoid masses, 2 to 6 cm. across, of gelatinous, greyish tumour with friable, opaque, yellow areas. (*b*) The amputated limb contained a single gelatinous tumour-nodule, 1·8×1·0×0·8 cm., deep to and invading the belly of the superior gemellus. Surgical scarring, and degenerative changes due to the nerve resection were present; the bones and joints were normal.

## HISTOLOGY OF THE TUMOURS

The material available comprised:—Case 1: Both specimens. Case 2: Amputated leg, and stained paraffin sections (hæmatoxylin and eosin; hæmatoxylin and van Gieson's stain) of four portions of the original tumour. Case 3: Gilmour's original paraffin blocks of one portion of the tumour removed in January 1943 and three of that removed in February 1943. Case 4: Three paraffin-imbedded portions of the 1946 specimen and two of the 1948 specimen; both 1949 specimens; 1950 specimen. The material was fixed in formol-saline (4 per cent. formaldehyde) in all cases.

The following histological account of the tumours in case 1 is equally descriptive of the appearances in cases 2 and 3. The original tumours in cases 1 and 2 were identical; the recurrence in case 2 and the two available tumours in case 3 were identical with the recurrence in case 1. The differences between the tumours in case 4 and those in case 1 will be described subsequently.

### *Histology of original tumour in case 1*

There is a thin, partly hyaline, collagenous capsule merging with underlying tumour tissue through a narrow, loose-textured zone containing groups of mature fat-cells. The tumour is composed of three varieties of tissue—vascular, fibrous and adipose. The vascular and fibrous components are intimately blended, forming lobules ranging from a few mm. to several cm. across, the interlobular areas being occupied by the less abundant adipose component. The vasculo-fibrous and adipose areas (fig. 2) in general are not sharply delineated, although in places completely separated by thick collagenous trabeculæ, sometimes with many elastic fibres. Occasionally there are large blood vessels in the trabeculæ, with no muscle or elastic elements in their thin walls. Their capillary branches anastomose with the neoplastic capillaries.

*Vasculo-fibrous component of tumour.* This resembles a fine sponge-work (fig. 3), the septa being formed by narrow, anastomosing capillaries. Most of the vessels are empty, their walls in contact and the lumen often incompletely formed; others contain normal blood. The endothelial cells are more prominent than in normal vessels,

MESENCHYMOMA

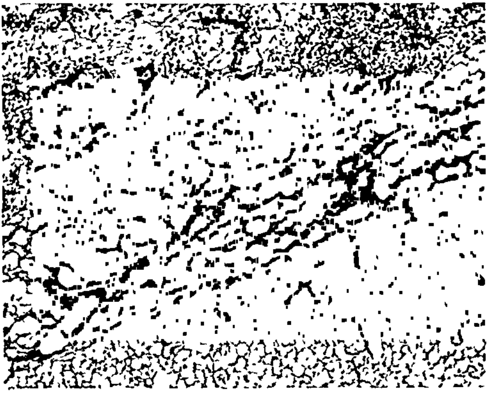


FIG 2.—Case 1. Original tumour. Adjacent zones of immature adipose tissue and capillary sponge-work separated by a broad fibro-fatty septum containing many large mature fat-cells. Phosphotungstic acid-hæmatoxylin. × 100.

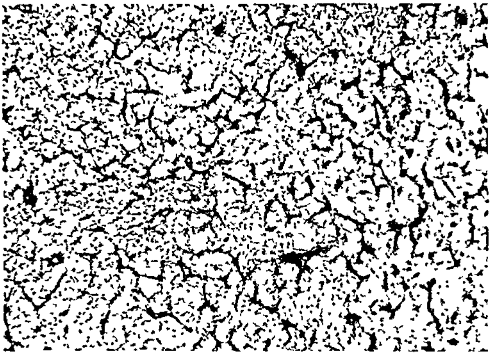


FIG. 3.—Case 1. Original tumour. Mesh-work pattern of neoplastic capillaries Phosphotungstic acid-hæmatoxylin. × 65.

MESENCHYMOMA

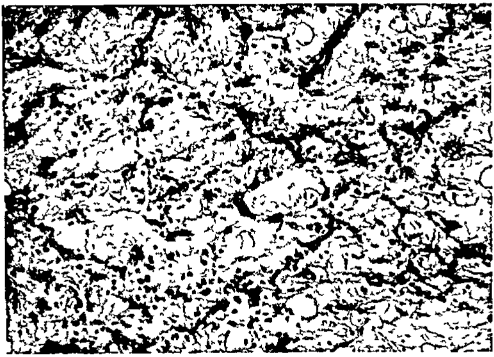
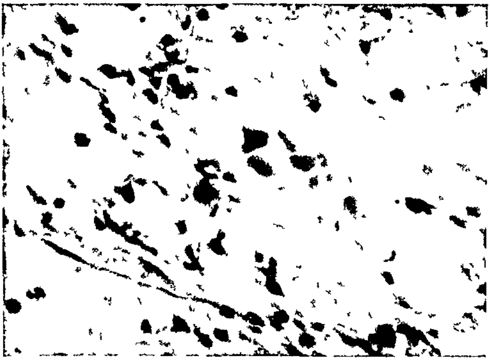


FIG 4—Case 1 Original tumour Capillary sponge work showing fibrillar structure in intercapillary spaces and scattered fat cells of different degrees of maturity Masson's tri chrome stain ×200

FIG 5—Case 1 Original tumour Part of a vascular nodule showing three cells near the centre resembling nucleated red blood cells and in their vicinity several globules of eosinophil hyaline material Hæmatoxylin and eosin ×400



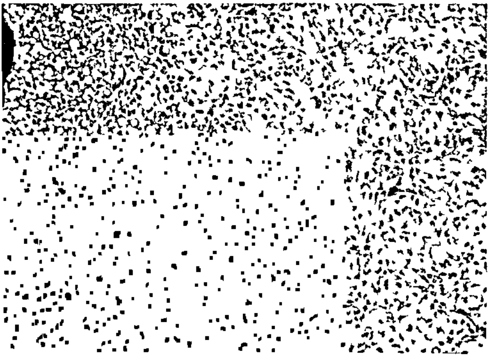


FIG 6—Case 4 Original tumour Junction between liposarcomatous area (on left) and vascular zone Hæmalum and eosin ×100

with more cytoplasm and longer, plumper nuclei. New vessels bud from those already formed, especially near the periphery of the lobules. Fine reticulin, collagen and elastic fibres lie external to the endothelium and radiate across the interstices of the sponge-work. Scattered ovoid, spindle-shaped or stellate cells are found in intimate contact with the fibres ; their faintly striated, acidophil or basophil cytoplasm is often united with adjacent cells by fine cytoplasmic processes. A few cells resemble smooth-muscle cells, having cytoplasmic fibrils which are stained deep blue by phosphotungstic acid-hæmatoxylin. Occasional fat-cells are present in the interstices, particularly towards the periphery of the vascular lobules ; the cells range from immature lipoblasts to fully adult cells (fig. 4). The capillary sponge-work is most cellular and of finest mesh in the outer parts of the nodules. Here, as well as fibroblasts and lipoblasts, there are some small, round or polyhedral, undifferentiated cells resembling the so-called undifferentiated mesenchymal cells of mature connective tissues. Occasional mast-cells are found, especially near the trabeculæ. Eosinophil leucocytes are scanty and there are no extravascular neutrophil polymorphonuclears. Scattered lymphocytes are present, and a few normal erythrocytes have escaped into the interstitium.

The interstices of the sponge-work are filled by a finely granular ground-substance which appears pale pink when stained with Mayer's hæmalum and eosin, pale blue with Ehrlich's hæmatoxylin and eosin and intensely metachromatic with 1 per cent. toluidine blue, and which has no affinity for neutral red by Duffett's (1949) hæmatoxylin-neutral-red technique, or for Southgate's mucicarmine. There are cysts in the interstices of the sponge-work in some areas, mostly about 200 μ across, although some are as much as 3 mm. in diameter ; some are partly or completely lined by barely visible flat cells, or by bigger cells with large, pale nuclei and much hæmatoxyphil cytoplasm which often contains acidophil, hyaline droplets ; some cells project on a narrow stalk into the cyst, or lie free within it. The contents of the cysts resemble the interstitial ground-substance ; in the smaller cysts the contents are only faintly metachromatic with toluidine blue, but have marked affinity for neutral red and for mucicarmine ; in the larger cysts the affinity for the latter stains is less or absent, and there is no metachromasia. Homogeneous, rounded masses are found in many of the larger cysts ; they are strongly eosinophil and are not metachromatic or stained by mucicarmine, although they have a marked affinity for neutral red ; they stain dark purple with phosphotungstic acid-hæmatoxylin, and with Heidenhain's iron hæmatoxylin they are more resistant to differentiation than erythrocytes ; they contain no demonstrable lipid ; they do not give a reaction for hæmoglobin by the Okajima-Dunn method (Lillie, 1948) or by the benzidine-nitroprusside-oxidase method of Lepehne and Pickworth (Lillie). Occasionally this material surrounds erythrocytes, as is well seen in trichrome (hæmatoxylin, Ponceau-acid-fuchsin, fast green) preparations, in which the erythrocytes stand out sharply from the surrounding deep green substance. One or more dense chromatin masses, 3 to 6 $\mu$ in diameter, apparently pyknotic nuclei, are present in some of the globular bodies in the cysts and in non-cystic areas (fig. 5); the smaller of these closely resemble pyknotic normoblasts, but most are of irregular shape, and their staining reactions do not confirm the morphological impression. Two other cell-types in the vasculo-fibrous areas morphologically resemble nucleated red blood cells. One occurs in cystic areas, the resemblance being close only at low magnification; this type contains an ovoid, reticulated nucleus and single or multiple, closely packed, homogeneous bodies of the general size and shape of erythrocytes; some resemble Russell bodies, but differ from the latter in staining more brightly, in completely losing the neutral red when stained by Duffett's method, in reacting positively to the Okajima-Dunn and Lepehne-Pickworth reagents, and in containing fine pigment giving a positive Perls's reaction for iron. Cells of the third type somewhat resemble normoblasts or megaloblasts, but the cytoplasm gives neither the Okajima-Dunn nor the benzidine reaction, and the nuclear structure ranges from pyknosis to that typical of histiocytes.

*Adipose component of tumour.* This is of simpler structure; mostly it is almost fully differentiated, but in places there is a considerable proportion of less mature cells, and in other areas nearly all cells are late lipoblasts. Most of the fat is sudanophil, with a small proportion of minute, needle-shaped, birefringent crystals, staining feebly, if at all, with Sudan dyes; the crystals are more abundant in areas of better differentiation. Blood vessels and connective-tissue fibres are scanty.

In all parts of the tumour mitoses are very rare and there are no abnormal mitotic figures. Necrosis is absent.

### *Histology of recurrent tumour in case 1*

The recurrent tumour is characterised by a considerably smaller proportion of adipose tissue, and by greater cellularity of all components, due partly to capillary proliferation as small, solid outgrowths from the vessel-walls, but mainly to the increased number of fibroblasts and small, round, undifferentiated cells. Mitoses are very rare. The main mass and the eight accessory nodules are of the same structure and each has a narrow, fibrotic capsule. The hyaline capsule of the main mass (case 1 only) contains a few small, ill-defined areas of chondroid structure, with patchy calcification of the matrix.

No tumour tissue was found in sections of any part of the amputated limb in cases 1 and 2, other than in areas macroscopically involved. In none of the first three cases of the series had the tumour broken through the capsule and there was no involvement of the thickened,

MESENCHYMOMA

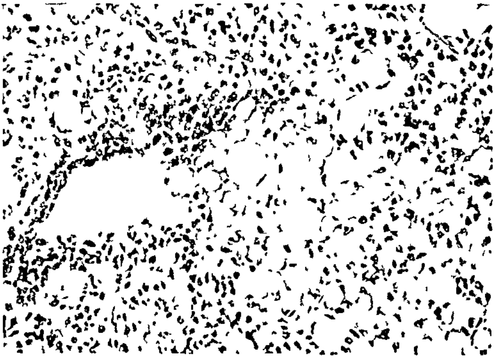


FIG. 7.—Case 4. Recurrence excised in July 1949. Island of immature adipose tissue adjacent to a small mucinous cyst surrounded by capillary meshwork. Hæmalum and eosin. ×200.

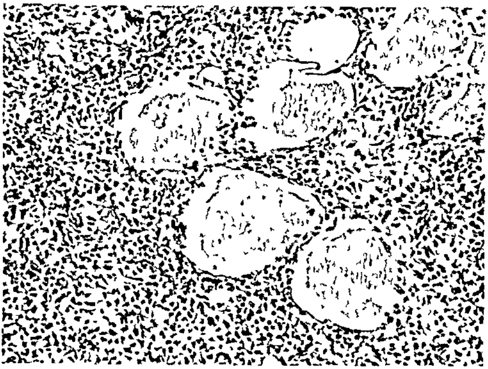


FIG. 8.—Case 4. Recurrence excised in July 1949. Group of mucinous cysts in cellular sarcomatous tissue. Hæmalum and eosin. ×100.

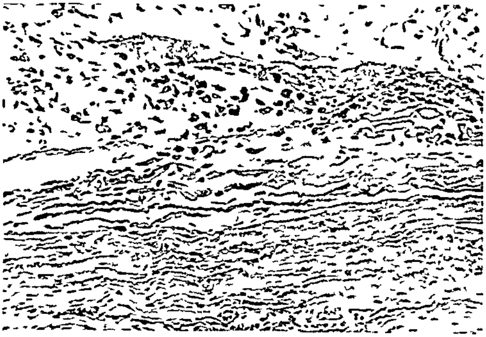


FIG. 9.—Case 4. Tumour nodule from amputated limb. Invasion of gemellus muscle. Hæmatoxylin and van Gieson's stain. ×200.

hyaline periosteum of the femur in case 2. In case 3 prolongations of the tumour into the muscles were completely separated from muscle tissue by intact capsule.

### *Histology of tumours in case 4*

The original tumour in case 4 and the recurrences differ only in the greater cellularity of the latter, in which mitoses are relatively more numerous. The basic structure of the tumours is the same as in case 1. In case 4 there are fewer mature cells in the adipose component (figs. 6 and 7). The vasculo-fibrous areas are much more cellular than in case 1, the intercapillary spaces being loosely packed with spindle-shaped and stellate cells (fig. 8); there are fairly numerous large, bizarre cells which contain one or more atypical, hyperchromatic nuclei; mitotic figures are fairly common and many are atypical. Unequivocally sarcomatous appearances are seen in both adipose and vasculo-fibrous areas and there are transitions between immature adipose tissue, liposarcoma and undifferentiated sarcoma, and between the tissues of the capillary sponge-work, fibrosarcoma and undifferentiated sarcoma. No vasoformative activity by sarcomatous elements is apparent. In places the structure is "myxosarcomatous"; such areas are distinct from the mucinous cysts. Cysts are numerous, their lining being more prominent than in case 1 and at times resembling hyperplastic synovia. Considerable areas of necrosis are seen. Encapsulation is incomplete, with areas of invasion of the surrounding tissues in all specimens. The nodule from the amputated limb (fig. 9) and the biopsy of the abdominal mass are similar to the other tumours. No tumour tissue is present in an external iliac lymph node removed during amputation, or in any part of the limb not macroscopically involved.

## DISCUSSION

### *Nature of the tumours*

All the tumours had the same basic structure, being composed solely of vascular, fibrous and adipose tissues of embryonic type. Chondroid foci, which occurred only in the capsule of the recurrent tumour in case 1, were thought to represent a metaplasia of the capsule and not to be neoplastic. The vascular and fibrous components were intimately blended, forming lobules between which lay the adipose component. The capillaries forming the vascular component were undoubtedly neoplastic; their number appeared greatly in excess of the nutritional needs of the tissues, and many were incompletely canalised; further, the peculiar sponge-work structure of the vasculo-fibrous areas appeared to have been determined by the pattern of growth of the vessels. Collagen, reticulin and elastic fibres formed an integral part of the tumour, being the product of differentiating tumour-cells. Fibroblasts and lipoblasts at various stages of differentiation and a few smooth-muscle cells occurred in or near the capillary walls. Stellate cells, as seen in myxomatoid tissues, and cells resembling the so-called undifferentiated mesenchymal cells of mature tissues were also present. Mucinous ground-substance filled the interstices of the sponge-work. Minute cysts containing similar material were partially lined by cells resembling mesothelial or synovial cells.

The tumours formed a graded series. The original tumours in cases 1 and 2 were well differentiated; the recurrences in these cases and the available tumours in case 3 were more cellular but still histologically benign; and in case 4, although all specimens contained areas identical in structure with those in the other cases, the original tumour was frankly sarcomatous and its recurrences were progressively more cellular and pleomorphic. The multiple recurrent growths in each case may indicate either multifocal neoplasia or the escape of viable tumour cells during operation; there is no convincing evidence of local lymphatic or hæmatogenous spread. Although the tumour in two cases developed close to the sciatic nerve, it could easily be freed from the nerve at the earlier operations and its origin from the nerve can be excluded.

It is suggested that these growths, three histologically benign and the fourth histologically malignant, are examples of a tumour that is peculiar in being formed of varied tissues, the non-neoplastic analogues of which are all embryonic derivatives of mesenchyme. Such a tumour could arise from differentiation, along more than one line, of undifferentiated cells that retain some of the pluripotentiality of the embryonic mesenchyme. The behaviour of all the tumours was that of a neoplasm of low-grade malignancy, displacing and compressing the surrounding tissues but invading them only in the case of the histologically sarcomatous growth. The tendency to local and progressively more cellular recurrence is more important than the absence of histological criteria of malignancy in assessing the character of the process in the first three cases.

### *Relation to liposarcoma*

Ewing (1935, 1940) distinguished two main types of liposarcoma. One consisted of fœtal fat-cells, myxomatous tissue and a " peculiar proliferating capillary vascular system ": this type he named fœtal or embryonal myxoliposarcoma and his photomicrograph (Ewing, 1940; fig. 51) shows a structure like that illustrated in fig. 2 of the present paper. Ewing described the second type as consisting of fat-cells like those seen in regenerating inflamed adult adipose tissue, with predominance of spindle-shaped and granular polyhedral cells, and more or less anaplasia; he called it adult liposarcoma. While recognising the variation in structure which may be seen in liposarcomata (Stout, 1944), we do not believe that the tumours in our cases are tumours of adipose tissue. The tumours usually designated liposarcomata (Ewing's "adult liposarcomata") do not show the peculiar organoid mixed structure which we have described; their pleomorphism is characteristically diffuse and circumscribed foci of differentiated tissues are not formed.

### *Hæmopoiesis in the tumours*

We found no evidence of hæmopoiesis in any of the tumours. We have been unable to confirm Gilmour's observation of immature blood cells in his case (our case 3), but we have seen only two of the five recurrent tumours available to him. Cells resembling nucleated red blood cells were present in all four cases in our series, but we interpreted them as phagocytes. Similar cells are commonly present in small numbers in a variety of normal and pathological tissues, *e.g.*, in the stroma of young chorionic villi, in myxomatoid tissue (neoplastic or degenerative) and in the matrix of the common pleomorphic salivary-gland neoplasms. The material in most of these cells was identical with that forming the hyaline masses in the cysts and present in some of the mesothelioid lining-cells. So-called "specific" staining reactions of hæmoglobin were given by other cells, but from their appearances and nuclear characteristics we believe that these were histiocytes which had engulfed free erythrocytes.

### *Nomenclature*

By describing these tumours as liposarcomata, Ewing (1935, 1940) implied a histogenesis which we consider disputable. Gilmour (1943) described the tumour as a teratoblastoma arising in a cell which still contained determiners for the formation of several kinds of mesodermal tissue; he suggested that it could be named mesenchymoma or connectivoma. Connectivoma, derived from the obsolete term *connectivum*, has nothing to recommend it. Gilmour used the name *mesenchymoma* because he considered that the tumour was formed of embryonic connective tissue, and Willis (1948) accepted it as appropriate because Gilmour's tumour "consisted of mucinous tissue like embryonic mesenchyme with also lipoblasts and hæmopoietic cells".

The earliest uses of the word mesenchymoma which we have been able to trace are by Klein (1932) and Cellina (1932). Klein defined mesenchymoma as a sarcoma of such primitive nature that only a more or less uniform cell-type is present, resembling the cells of undifferentiated mesenchyme or at most differentiated only to the point of blood-channel formation. He applied the term to Ewing's tumour of bone, angiosarcoma and some endotheliomata, and said that where tumour cells resemble differentiated cells or produce, for example, fibrous tissue or bone, one is no longer dealing with mesenchymoma but with entities such as fibrosarcoma or osteogenic sarcoma. Cellina used the name in a wider sense; he reviewed the non-epithelial hepatic tumours, grouping them all as mesenchymomata.

Marburg (1934-35) described as a meningeal mesenchymoma a tumour formed of undifferentiated and fibroblastic cells at the site of an iodoform-gauze swab left *in situ* during partial excision of an astroblastoma 5 years earlier. Tauber *et al.* (1938) described an invasive, metastasising " turban tumour " composed of large cells with argyrophil cytoplasmic processes; they called it an " undifferentiated malignant tumor of mesenchymal origin, a mesenchymoma". They suggested that this name was valuable as designating a new class in which to place tumours of doubtful nature, thus avoiding their unjustified inclusion in the categories sarcoma and carcinoma and taking many tumours of uncertain nature out of the category " endothelioma."

Takács (1942) reported a metastasising uterine " myxo-angio-endothelioma " ; he suggested that it originated from mesenchymal parts of retained chorionic villi, and named it mesenchymoma embryonale angioplasticum. Stout (1948) (*infra*) accepted this case as a mesenchymoma, but erroneously referred to it as benign. Novak (1947) grouped granulosa-cell tumours, thecomata and " luteomata " together as ovarian mesenchymomata, on the grounds that granulosa and theca develop from ovarian mesenchyme; to distinguish them from dysgerminoma, which he said is derived from ovarian mesenchyme " in its undifferentiated phase ", that is, from cells not yet definitely stamped with feminine potentialities, he suggested the expression " feminizing mesenchymoma of the ovary " for the former group. The still uncertain genesis of normal and neoplastic ovarian elements makes Novak's terminology inadmissible; moreover, if the ultimate mesenchymal origin of a cell were to entitle its neoplastic derivatives to the name mesenchymoma, this term could cover most non-epithelial neoplasms and some epithelial ones.

Stout (1948) defined the mesenchymomata as a group of neoplasms in which two or more tissues of mesenchymal derivation participate and are *amalgamated* into a single mass. He recognised benign and malignant varieties, and said that they occur most often in certain regions where congenital malformations are common (urogenital organs, breast). Amongst the benign forms Stout included hamartoma of the lung, arbitrarily excluding those containing *epithelial* elements, and several other conditions which also appear to be hamartomatous although he did not so describe them. Stout classified as malignant mesenchymomata a variety of tumours each containing connective tissues of more than one type, any one of which had the structure of a primary sarcoma. He deprecated the term mixed tumour for such growths because of its association with tumours of salivary and similar glands and with urogenital and mammary tumours containing epithelial and connective-tissue elements. Mesenchymoma appeared to him a safer and less clumsy term than mesodermal or mesenchymal mixed tumour. None of Stout's (1947; 1948) malignant mesenchymomata was identical in composition with the tumours reported in the present paper, but there is no doubt that the tumours in each series are of the same nature and are the product of neoplastic growth of cells with a mesenchyme-like capacity to form a variety of tissues. Stout's tumours were more complex due to differentiation along additional lines. The retroperitoneal " mixed tumours " reviewed by Lähteenmäki (1945) are similar.

This review shows that there is confusion in the use of the name mesenchymoma. If the name is to be used, we believe that it should be given only to neoplasms composed of more than one type of connective tissue, the structure of which is that of differentiating embryonic tissues which develop from and replace the primitive mesenchyme. The tumours reported here could be so named.

### *Prognosis and treatment*

These locally malignant tumours are characterised by comparatively slow initial growth and local recurrences at progressively shorter intervals after simple excision of the apparently encapsulated masses. Successive recurrences tend to be more cellular and less well differentiated. Distant metastases did not develop, so far as is known, in the two surviving patients in our series, although their occurrence as a relatively late event in the history of tumours of this type would not be surprising. In the patients who died (cases 3 and 4) there was no clinical or radiological evidence of metastasis a few weeks before death, but in the absence of post-mortem examination metastasis cannot be excluded. Blood-borne metastases developed in a few of the more complex, frankly sarcomatous, tumours in Stout's (1948) series.

With regard to treatment we believe that there should be no avoidable delay in performing amputation if recurrence follows local excision. This should be done even when the tumours appear histologically benign, for subsequent recurrences are likely to show more active growth and the possibility of eventual metastasis cannot be excluded. The tendency to grow along fascial planes makes it necessary to choose the site of amputation so that an adequate margin is left well above the level of the clinically determinable limits of the growth. If amputation is impracticable a combination of excision and deep X-ray therapy offers the best chance of preventing recurrence and, in the event of recurrence, of postponing death. Excision should be undertaken with particular care to prevent rupture of the tumour and seeding of viable tissue in the wound ; if biopsy is performed the biopsy wound should be widely excised when the tumour itself is removed.

## SUMMARY

Four cases of an unusual recurring connective-tissue neoplasm are described. The tumours consisted of immature vascular, fibrous and adipose tissues resembling the embryonic connective tissues which develop from the undifferentiated mesenchyme. No evidence of hæmopoiesis was found in any of the four cases although cells resembling nucleated precursors of erythrocytes were seen ; they were interpreted as phagocytes.

In one case the tumours were frankly sarcomatous in structure ; in the others they were histologically benign. This is considered to be of little importance in relation to prognosis, as local recurrence developed in all cases. Metastasis was not observed in any of the cases, although its development would not be surprising.

Two of the patients were men and two were women. The ages at which the tumour was first noticed ranged from 23 to 47 years, and the durations before excision from 8 weeks to 3 years. The intervals between the first excision and the first recurrence ranged

from 13 months to more than 4 years (18 months for the histologically sarcomatous growth). In three cases amputation was necessary 35, 37 and 52 months respectively after the tumour was first noticed. In the third case the patient died with intra-abdominal extension of the tumour little more than a year after hind-quarter amputation. In the fourth case the tumour recurred repeatedly over a period of 24 years despite surgical and deep X-ray therapy; the recurrent tumours were moderately radiosensitive.

Treatment by amputation is advised if recurrence follows local excision. If amputation is impracticable recurrence should be treated by a combination of excision and radiotherapy.

We wish to thank Mr W. B. Foley (Oxford), Mr G. H. Marshall (Worcester), Mr R. W. Reid (Colchester) and Mr B. N. Brooke (Birmingham) for permission to record cases 1, 2, 3 and 4 respectively; Dr J. A. C. Fleming (St. Thomas's Hospital, London) and Dr J. F. Bromley (Birmingham) for details of the radiotherapy in cases 3 and 4 respectively; Dr R. H. Cowdell (Oxford) for the macroscopic description of the amputated limb in case 1, Dr P. Kidd (Worcester) for the amputation specimen and stained sections in case 2, and Dr J. B. Penfold (Colchester) for the paraffin blocks in case 3; Prof. J. W. Orr and Dr A. H. T. Robb-Smith for their interest; and Dr G. A. C. Summers (Bristol) for the photograph of the specimen in case 1.

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# THE TAKING AND PROJECTION OF STEREOSCOPIC PHOTOGRAPHS IN COLOUR OF SMALL OBJECTS

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(PLATE LXXIV)

THE viewing of a stereoscopic picture is no longer restricted to one person at a time, and the description of a relatively cheap method of taking and projecting stereoscopic pictures in colour of small objects may now prove of interest to many workers in various branches of biology.

During the course of research on lung structure and development, which is still in progress, the writer has developed a technique for the preparation of neoprene latex casts of the air spaces and for the study of the surrounding lung tissue (Morison, 1948). The structures to be studied are three dimensional and may be in various colours. They are dissected and studied under the binocular dissecting microscope and ordinary photography fails to provide a satisfactory record. The casts tend to become brittle on storage and it is also necessary to retain a record of casts at various stages of dissection. The material is usually dissected under water and no system of stereoscopic photography requiring movement of the object is possible. For this and other reasons methods usually available for the taking of stereoscopic photographs at various magnifications are not suitable. These methods, and methods of projecting stereoscopic transparencies so that they can be observed by a number of people at once, are reviewed by McKay (1948) and Judge (1950). The apparatus designed has proved convenient to the author both for its original purpose and in a study of placental structure and even for the photography of small specimens. However, it is not claimed that the principles involved are in any way novel.

## THE STEREOSCOPIC CAMERA

*Object stand.* This must be the stage of the twin objective binocular microscope used for the dissection and study of the specimen (fig. 1). This stand is so fixed that an object seen in the centre of the field of the microscope is also centrally positioned for any lens advanced over it by the camera. The specimen is usually lit by incident light from an enclosed 6 volt 30 watt lamp fitted with focusing bull's eye from 13 months to more than 4 years (18 months for the histologically sarcomatous growth). In three cases amputation was necessary 35, 37 and 52 months respectively after the tumour was first noticed. In the third case the patient died with intra-abdominal extension of the tumour little more than a year after hind-quarter amputation. In the fourth case the tumour recurred repeatedly over a period of 24 years despite surgical and deep X-ray therapy; the recurrent tumours were moderately radiosensitive.

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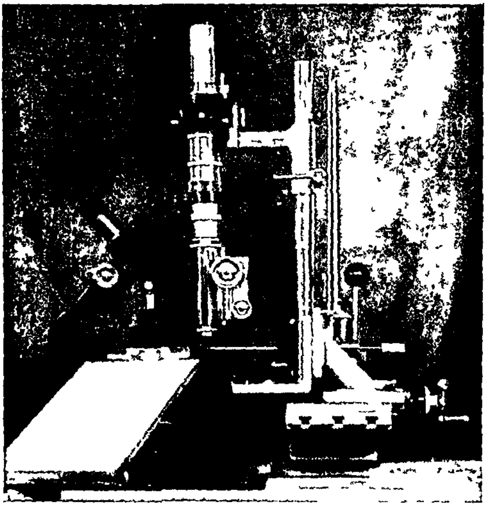
## PLATE LXXIV

FIG. 1.—The object is dissected in water on the stage of the twin objective binocular microscope. The stereoscopic camera is seen occupying more than the right half of the picture. It is not in use. Its lens system has been focused so that the point of the needle appears sharp on the clear glass screen of a Stewartry Leica copier. The needle is seen to pass through the spindle of the swivelling movement of the milling slide. The ball handle for locking the rotary movement of the swivelling milling slide is also seen.

FIG. 2.—The body of the binocular dissecting microscope has been removed and the entire camera advanced over the object. The handle of the worm movement responsible for this horizontal advance is seen in the extreme right lower part of the picture. The slide for focusing by raising or lowering the whole camera is at right angles and the handle for its worm movement is seen just above the focusing needle, which is now almost completely withdrawn.

STEREOSCOPIC PHOTOGRAPHS



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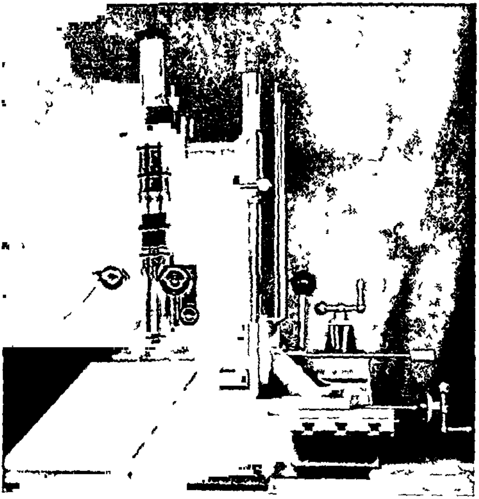

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movement provided on the swivelling milling slide (fig. 2). Because the position of the binocular stand was previously fixed this horizontal movement advances the centre of the camera lens over the centre of the field studied by the binocular dissecting microscope. Actually, even if the stand of the binocular dissecting microscope did shift and the state of the dissection did not permit its movement back into position, the camera could be moved to one or other side. A second rotary movement about a vertical spindle is provided on the swivelling milling slide and this could be unlocked for this purpose.

*Focusing the camera on the object.* The camera is focused on the object by raising or lowering the whole camera and leaving the lens system, which has been focused on the centre of rotation of the camera, untouched. This is easily done since the entire camera, consisting of lens system, copier and camera body and swivelling milling slide, is carried on the second, or plain vertical milling slide (fig. 2). The worm movement this provides gives a vertical movement which is satisfactory for all but magnifications of a hundred or over. The frosted glass of the copier has been replaced by clear glass with a fine engraved scale on it and the image is focused on this and viewed through a suitable focusing telescope.

*Taking the pair of stereoscopic pictures.* One picture can be taken with the camera directly over the object. The other must be taken after the camera has been rotated to one or other side by an amount which will be discussed later. A simple pointer and a scale marked in angles are carried on the moving and on the fixed components of the swivelling milling slide and measure the rotation. After the first picture has been taken the second is obtained by releasing the rotary movement of the swivelling milling slide, rotating it to right or left as desired and re-locking it. Re-focusing of the object should not be necessary.

## DETERMINATION OF EXPOSURE TIME

Exposure is highly critical in colour photography and it is very difficult to standardise incident lighting of the object. Instead the useful high and low light values from different parts of the image as it appears on the clear glass focusing screen are determined. This can be done with considerable accuracy with a SEI photometer (Messrs Ilford Ltd.). A simple pocket lens fitted over the viewing lens of the photometer allows the image on the clear glass focusing screen to be seen. The instrument superimposes a small and diffusely illuminated light spot on the image and this is matched against the brightness of the image. The brightness of the light spot can be modified by photometric wedges and filters in the instrument and the intensity of the light source is accurately controlled. The brightness of any portion of the image can be expressed in foot-lamberts or in log foot-lamberts, but the former is sufficiently accurate. Using

Kodachrome Type A film the exposure time in seconds, when the useful low light is measured, will probably be 10 to 15 divided by the light value in foot-lamberts. If the high light is measured it may be 20 to 30 divided by the reading in foot-lamberts.

## STEREOSCOPIC PROJECTION

*Principle.* The left-hand picture must be seen with the left eye and the right-hand picture with the right eye of the observer. This can be done if the two pictures are each projected through separate projectors. The light of these two projectors is polarised and this polarisation is so arranged that the plane of polarisation of the light of one projector is at right angles to that of the other. The left-hand picture is projected by light which, because of its plane of polarisation, is entirely, or almost entirely, obliterated as it passes through a polaroid film held in front of the observer's right eye. This light readily reaches the left eye because the polaroid film in front of that eye is polarised in the same plane as the polaroid screen polarising the light of the left-hand projector. Similarly light from the right-hand projector reaches the right eye, but cannot pass the polaroid film in front of the left eye with its plane of polarisation at right angles to the polariser in the right-hand projector. The light received by the observer is reflected from a screen and it is obvious that this screen must not depolarise the light. A silver metallic-painted screen is suitable, but a white screen cannot be used.

*Arrangement of projectors.* Two Aldis 35 mm. projectors with 100 mm. projector lens are used, but other projectors could be used. In these projectors it is easy to fit polariser screens on to the lens mount so that they lie between the slide carrier and the projector lens. A circular disc of polaroid is placed between two flat brass rings which are attached to one another by screws. One ring is slightly larger than the other and is held against the flat surface of the lens mount by three screws at its edges. It is rotated until the light from this projector is obscured to the maximum degree as seen by the opposite eye through the polariser spectacles to be employed. The screws are then tightened and the screen securely locked in position.

The projectors are placed close to each other. Each is focused separately on the screen. It has not been found useful to complicate the arrangement by fixing the projectors to a base. When the two pictures are projected simultaneously on the screen and viewed without suitable spectacles they appear as a confused jumble. The transparencies are rarely so accurately mounted that objects in both appear at the same height from the bottom. It would be very difficult to correct this by any positioning of the transparencies in their mounts, but it can be easily corrected by raising or lowering one of the projected images, usually the right, by adjusting the two positioning screws provided on the front of the projector. By a slight movement of one projector or slide carrier to one or other side the two pictures can then be brought into coincidence at one point. It is usually convenient to bring the two images of some distinct object lying in the plane nearest to the observer into coincidence. If the picture is then viewed with the stereoscopic spectacles it should appear three-dimensional and lying behind the screen. These adjustments can be made rapidly by glancing over the top of the spectacles. Both images are then seen and prominent objects can be selected and the pictures brought into coincidence.

*Position of screen and spectators.* It will usually be most convenient to arrange when taking the photograph that the projector-to-screen distance will also be the optimum viewing distance. In theory only one viewing position is correct and this must be arranged at the time of taking the picture. Even in stereoscopic pictures of familiar objects considerable variation in the distance is permissible and no distortion will be appreciated. In the photography of microscopical objects, the relative size and position of objects are necessarily unfamiliar and unknown distortion may not be so readily appreciated. Viewing positions too far from the screen tend to increase the antero-posterior diameter of objects and observation too near the screen tends to make objects appear flattened out.

## SEPARATION OF TAKING LENS

In orthostereoscopy all objects appear to the observer to be the same size as they were at the time of photography, and all appear to be situated at the same distance from him and from each other as they did at that time. Here the stereoscopic pictures are taken with lenses separated to the same extent as the eyes of the observer and are viewed by lenses identical with those used when photographing them. When these conditions are departed from very difficult and controversial problems arise but these cannot be discussed here. Wright (1938) has pointed out in a discussion of stereophotomicrographic methods that the visual angle subtended by the enlarged image is settled once for all by the magnification of the optical system. The effect of varying the separation of the lenses of the camera is to present the object as if it were seen from various distances. Thus it may appear the size of a match box a few inches from the observer, or with less lens separation as an object the size of a large book at ten feet away, or with very little separation as a suitcase at fifty feet. The assumption that a desirable viewing distance is 10 inches is entirely arbitrary. In microscopic objects of some depth this near viewing point, and the separation of lens necessary to attain it, may introduce considerable distortion, and, especially in projected transparencies, the objects often appear unreal. The image created may be compared to an enlarged model and it is often better to view this as if it were a large object at some distance rather than a smaller object closer at hand. For almost all small objects suitable for stereoscopic pictures the separation obtained by rotating the lens system through three or five degrees is sufficient to give adequate stereoscopic relief. Often even less will be sufficient and more will only occasionally be useful and then only for flattened objects or thin sections. If objects are to be compared the separation must be the same and it is probably better to employ only a few variations in viewpoint.

For objects about natural size on the film and for objects, such as pathological specimens, photographed at less than ten feet the separation should really depend on the depth of the field it is desired to render stereoscopically. Formulæ, such as those given by Judge, can be used, and must often also be corrected for a difference in the focal lengths of projector and taking lenses. It is probably more convenient to employ only a few object-to-camera distances and to standardise the lens separation. At this distance distortion can easily be introduced and unfamiliar objects may be incorrectly interpretated in the absence of a far distance plane of reference such as is usually present in orthosteroscopic pictures.

## Summary

A method is described for taking stereoscopic pictures of small objects in colour and for projecting these so that they retain their three-dimensional appearance. Controlled camera movement is obtained by mounting the camera with its lens system on swivelling and plain vertical milling slides of a lathe and the object itself is not moved. Projection employs two projectors with polariser screens and polarised viewing spectacles.

A grant from the Medical Research Council has made this work possible. Assistance has also been obtained from a grant from the Northern Ireland Hospitals Authority for Fœtal and Neonatal Research. Mr D. McC. Mehaffey advised on the use of lathe parts and machined the other components. Assistance from Messrs Polarizers (U.K.) Ltd. and Myford Engineering Company is acknowledged.

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516 . 858 . 23 (Columbia SK)

# OBSERVATIONS ON COLUMBIA SK VIRUS

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(Plates LXXV and LXXVI)

Columbia SK virus was first isolated by Jungeblut, Feiner, and Sanders (1942*a*) during experiments in rodents with the SK poliomyelitis virus, a strain of poliomyelitis virus which appears to belong to the Lansing group since it has been adapted to cotton rats and mice. Columbia SK virus is pathogenic for cotton rats, mice, golden hamsters *Mesocricetus auratus*, guinea-pigs (Jungeblut, Sanders and Feiner, 1942*b*) and for monkeys (Verlinde *et al.*, 1950).

There is now considerable evidence to show that Columbia SK is one of a number of viruses which together make up the encephalomyocarditis group. The other members of this group are the MM virus, the Mengo encephalomyelitis virus, and the EMC virus. The MM virus was isolated by Jungeblut and Dalldorf (1943) from the brain of a golden hamster which died 19 days after inoculation with material from the medulla and cord of a patient with poliomyelitis. The original human material was not pathogenic for cotton rats, mice or guinea-pigs, but after passage through the hamster the virus recovered was pathogenic for rodents. Schatz and Plager (1948) found that SK Columbia virus and MM virus cross-immunised, a result confirmed by Powell and Jamieson (1948) who also showed that both viruses could be propagated in the developing chick embryo.

The EMC virus, which produces myocarditis and encephalomyelitis in mice, was isolated by Helwig and Schmidt (1945) by the intracerebral inoculation of pleural fluid and spleen from a chimpanzee which had died with symptoms of bilateral hydrothorax, pulmonary œdema, pericardial effusion, and myocarditis. Warren and Smadel (1946, 1948), and Smadel and Warren (1947), as a result of studies on the serological, pathological, and physical properties of EMC virus, believed that it was closely related immunologically to the MM and Columbia SK viruses. In the sera of American soldiers who, while stationed in the Philippine Islands, had suffered from a short febrile illness in 1945 and 1946, Smadel and Warren demonstrated immune bodies against the EMC virus. Schmidt (1948) described myocardial changes in rodents inoculated with the EMC virus: interstitial infiltration of the interalveolar septa and, in a small proportion of animals, bronchopneumonia were also noted.

In Uganda, Dick, Smithburn, and Haddow (1948) isolated from a paralysed monkey the Mengo encephalomyelitis virus which is pathogenic for man, rhesus and cynomolgus monkeys, mice, and guinea-pigs. The virus is transmissible by certain mosquitoes such as *Taeniorhynchus* sp. (Dick *et al.*, 1948 ; Dick, 1948). Observations by Dick (1949) and Warren, Smadel, and Russ (1949) show that Mengo encephalomyelitis, encephalomyocarditis (EMC), Columbia SK, and MM viruses are very closely related serologically, in their pathogenicity and in their experimental host range. On the other hand, no immunological relationship was found between the encephalomyocarditis group of viruses and either poliomyelitis virus or spontaneous mouse encephalomyelitis viruses.

## THE RANGE OF HOST SUSCEPTIBILITY TO COLUMBIA SK VIRUS

The following observations were made during the course of experiments carried out to determine whether certain animals would be suitable for interference experiments between the Lansing strain of poliomyelitis virus and Columbia SK virus.

### *The susceptibility of hedgehogs*

The European hedgehog *Erinaceus europaeus* L. is known to be readily susceptible to yellow fever and to foot-and-mouth disease. Its susceptibility to Columbia SK virus was tested by intramuscular, subcutaneous, and intracerebral inoculation, by intranasal instillation and by rubbing the virus on to the intact skin of the abdomen. The inoculum was either a 1 in 100 suspension of infected mouse brain or blood. From table I it will be seen that Columbia SK virus was pathogenic when inoculated by all these routes.

TABLE I

*Infection of hedgehogs with Columbia SK virus*

| Animal no. | Route of inoculation | Inoculum | *No. of days from* inoculation till death |
|---|---|---|---|
| 1 | Intramuscular | 0·5 ml. (1 in 100) | 3 |
| 2 | ,, | 0·5 ,, ,, | 4 |
| 3 | ,, | 0·5 ,, ,, | 4 |
| 4 | Subcutaneous | 0·5 ,, ,, | 2 |
| 5 | ,, | 0·5 ,, (1 in 100 brain) | 2 |
| 6 | Intranasal | 0·5 ,, ,, | 3 |
| 7 | Intracerebral | 0·1 ,, ,, | 3 |
| 8 | ,, | 0·1 ,, ,, | 4 |
| 9 | Unbroken skin | 0·2 ,, mouse blood | 7 |
| 10 | ,, | 0·2 ,, ,, | 6 |

All the hedgehogs developed tremor and weakness, especially in the hind legs, and failed to roll up when touched. Most of the animals were killed when moribund and blood and saline suspensions of brain, liver, and kidneys were inoculated intraperitoneally into mice. Virus was present in blood, brain and other organs of the hedgehogs as shown by the death of the inoculated mice in two to three days. In many hedgehogs when moribund the bladder was greatly distended, but virus was not found either in urine or in stools by the inoculation of mice. Virus from hedgehogs transmitted to mice was neutralised by an antiserum prepared in the rabbit against Columbia SK virus. In addition, the virus after transmission through hedgehogs was

COLUMBIA SK VIRUS

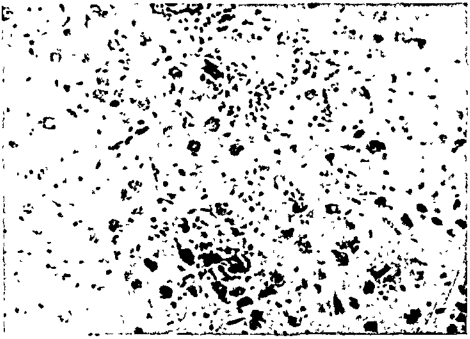
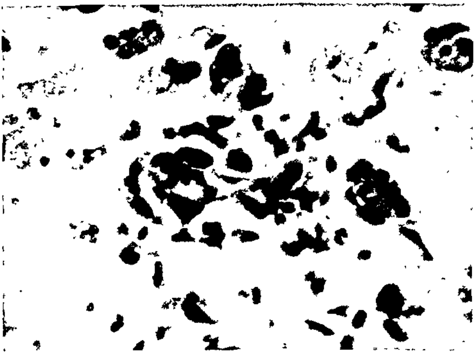


FIG. 1.—Mid-brain of hedgehog, infected with Columbia SK virus, with areas of diffuse infiltration and neuronophagia. Hæmatoxylin and eosin. ×150.

FIG. 2.—Mid-brain of hedgehog, infected with Columbia SK virus, showing neuronophagia, degeneration of neurones and early perivascular changes. H. and E. ×550.



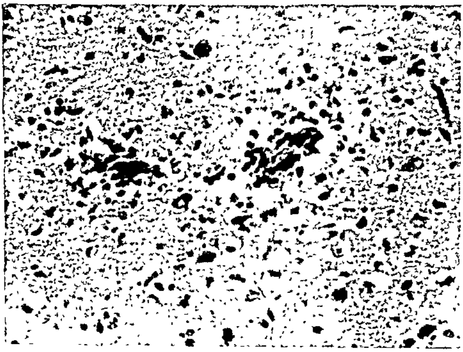


FIG. 3.—Medulla of mouse infected with Columbia SK virus showing similar changes to those in hedgehog brain. H. and E. ×400.

COLUMBIA SK VIRUS

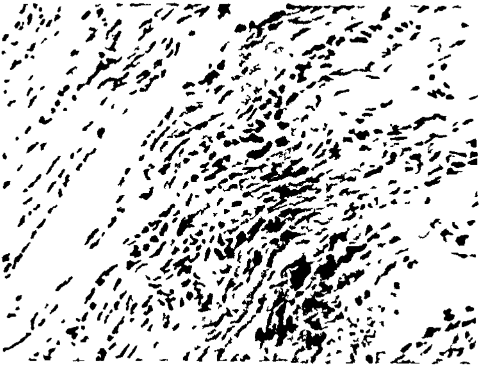
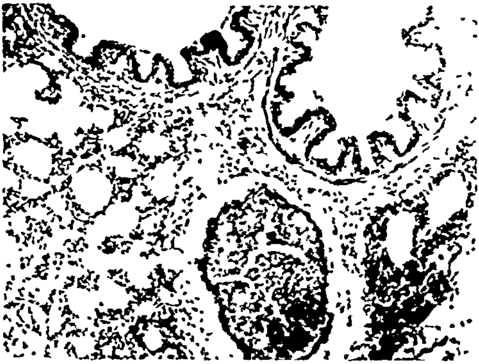


FIG 4.—Heart muscle of hedgehog infected with Columbia SK virus showing acute myocarditis H and E ×250

FIG 5.—Lung of hedgehog infected with Columbia SK virus showing congestion and infiltration of the alveolar walls H and E ×150



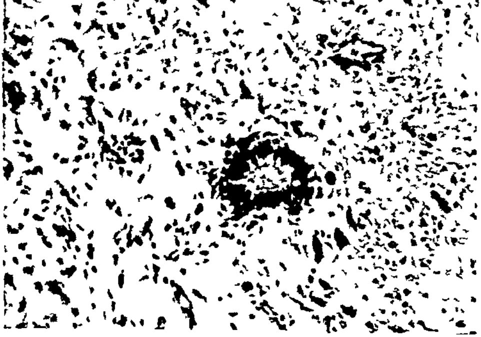


FIG 6.—Cervical cord of 5 day old chick infected with Columbia SK virus showing diffuse and nodular infiltration H. and E ×400

neutralised in intraperitoneal tests in mice by an antiserum prepared in monkeys against the Mengo encephalomyelitis virus: 10,000 M.L.D. of virus were neutralised. On the other hand, this serum showed no neutralising action against Lansing poliomyelitis virus. The virus obtained from hedgehogs is thus presumably the Columbia SK virus and not an extraneous virus normally present in the European hedgehog. That the virus was not originally present in the hedgehogs employed is shown also by the fact that after inoculation of Lansing poliomyelitis virus intracerebrally into hedgehogs it was not possible to demonstrate Lansing or any other virus in the cord when the animals were killed from 3 to 15 days later and 0·03 ml. of a 1 in 10 saline suspension of cord was inoculated intracerebrally into mice; nor could histological changes be found in the hedgehog cord.

The pathological changes found in hedgehogs after inoculation with Columbia SK virus are very similar to those produced in other species by this virus. At post-mortem examination the only macroscopic change was areas of congestion in the lungs in some animals. Histologically the main lesions were present in the central nervous system and in the heart. In the brain and cord there was infiltration of the meninges and a slight degree of perivascular cuffing with lymphocytes and mononuclear cells. The most marked changes were seen in the mid-brain, medulla, and cervical cord. Degenerative changes were present in neurones, accompanied by intense neuronophagia and an increase in small round cells (figs. 1 and 2). These changes were irregularly distributed through the grey matter, giving rise to scattered foci of degeneration and infiltration, not unlike those found in the guinea-pig brain in louse-borne typhus. Very similar foci are found in the mid-brain and cord of mice infected with Columbia SK virus (fig. 3). No intranuclear inclusions were seen.

Myocardial changes were found in those hedgehogs which lived four days or longer. The changes were very similar to those described by Schmidt in guinea-pigs and by Verlinde *et al.* in the cynomolgus monkey. In the heart muscle and epicardium there was considerable lymphocytic infiltration, more intense in some areas than in others. In the more marked areas of infiltration the muscle fibres stained more intensely with eosin and in places the fibres were breaking up (fig. 4).

In the lungs there was congestion of the blood vessels, the interalveolar septa showed considerable infiltration with round cells and in some areas the alveolar spaces were dilated. The bronchi were free from exudate (fig. 5).

In those hedgehogs which lived four or more days, the liver showed scattered areas of round-cell infiltration with prominence of the Kupffer cells: early necrotic changes were present in some of the parenchymatous lesions. The other organs appeared normal.

### *Hedgehog fleas and Columbia SK virus*

As the hedgehogs were heavily infested with fleas an attempt was made to see whether these fleas could carry Columbia SK virus. The fleas, kindly identified for us by Dr Karl Jordan, F.R.S., as *Archaeopsylla erinacei* Bouché 1935, were collected from a hedgehog showing symptoms of illness 4 days after intramuscular infection of virus. The fleas were killed with ether, washed twice and ground up in sterile saline to form a 1 in 20 suspension. This suspension was inoculated intraperitoneally in 0·1 ml. doses into mice. The mice died in from 2 to 7 days with symptoms and histological changes in the brain similar to those produced with Columbia SK virus. After further passages in mice the flea-transmitted virus was neutralised by a Columbia SK antiserum prepared in the rabbit. Although Columbia SK virus is capable of being taken up from the blood of hedgehogs by the flea *A. erinacei*, it is apparently not transmitted by the bite of these fleas. Infected fleas from a hedgehog dying from Columbia SK infection were placed on a normal hedgehog which lived without showing any signs of infection for 21 days: it was then inoculated subcutaneously with mouse-brain suspension infected with Columbia SK virus and died in 3 days. When infected hedgehog fleas were crushed and then rubbed on to the abdominal skin of a hedgehog, death occurred in 7 days, the virus being present in the blood and brain.

### *The susceptibility of voles*

In addition to hamsters, cotton rats, guinea-pigs, and mice, the dormouse *Myoxus glis*, when inoculated intracerebrally with Columbia SK virus, has been shown by Sanz Ibáñez (1946) to die in from 3 to 8 days with paralysis. Orkney voles, *Microtus orcadensis*, were inoculated intracerebrally and intramuscularly with a suspension of mouse brain infected with Columbia SK virus. The virus was instilled intranasally, given in the food, and placed on the normal skin of the abdomen. The results are shown in table II.

TABLE II

*The infectivity of Columbia SK virus for the Orkney vole,* Microtus orcadensis

| Route of inoculation | Inoculum | No. of days from inoculation to death |
|---|---|---|
| Intracerebral . . | 0·03 ml. 1 in 100 mouse-brain suspension | 2, 2, 2, 3 |
| Intramuscular . . | 0·1 " " " " | 2, 3 |
| Intraperitoneal . . | 0·1 " " " " | 2, 2, 2, |
| Intranasal . . . | 0·02 " " " " | 3, 3, 4 |
| In food . . . | 0·1 " " " "<br>(added to mashed carrots) | 2, 2 |
| Placed on normal skin . | 0·1 ml. 1 in 100 mouse-brain suspension | 3, 4 |

Blood and brain from voles were inoculated into mice intracerebrally, intraperitoneally, and intranasally. Mice died after intracerebral inoculation of 0·03 ml. of a 1 in 100 vole-brain suspension in 2 days, after intraperitoneal inoculation of 0·1 ml. of the same suspension in 2 to 4 days, and after intranasal instillation in from 5 to 10 days. The mice dying in 8 and 10 days showed paralysis of the hind legs. The heart bloods of voles were bacteriologically sterile, as were the bloods of mice dying after inoculation with vole material. The virus, after transmission through voles, was neutralised by a Columbia SK rabbit antiserum. An attempt to transmit Lansing poliomyelitis virus to voles failed to produce any paralysis. The Lansing virus could not be recovered from the brain or cord more than 96 hours after intracerebral injection.

### *The susceptibility of albino rats*

In view of the possibility that the wild rat may form a reservoir for Columbia SK virus, an attempt was made to determine the length of survival of the virus in the rat. Jungeblut and Sanders (1940) had shown that virus could be detected in the brain, cord and liver of a rat 96 hours after intracerebral inoculation. In the present series of experiments, young albino rats weighing about 80 g. were inoculated intracerebrally with 0·05 ml. of a 1 in 100 suspension in saline of mouse brain infected with Columbia SK virus. Rats were killed after intervals of 24 hours, 3, 5, 7, 9, 13, 20 and 27 days; from the brains 1 in 100 suspensions in saline were prepared; 0·2 ml. was inoculated intraperitoneally into mice. The results are shown in the table.

TABLE III

*Intraperitoneal inoculation of mice with rat-brain suspension to demonstrate survival of Columbia SK virus*

| Interval from inoculation of rats to death (days) | Deaths of mice inoculated with rat-brain suspension (days) |
|---|---|
| 3 | 4, 4, 6, 6 |
| 5 | 4, 5, 6, 6 |
| 7 | 6, 7, 8, 8 |
| 9 | 6, 8, 8, 12 |
| 13 | 15, 15, S, S |
| 20 | S, S, S, S |
| 27 | S, S, S, S |

S = Survival

The results show that Columbia SK virus can survive for 13 but not 20 days in the brains of rats following intracerebral inoculation.

When rats were similarly inoculated intracerebrally with mouse cord infected with Lansing poliomyelitis virus, the virus could not be demonstrated for more than 48 hours.

### *Infectivity for chickens*

Columbia SK virus can be readily propagated in the developing hen's egg (Enright and Schultz, 1948), when the embryos are from 5 to 14 days old but not when they are more than 15 days old. Attempts have been made to adapt MM virus to chickens of various ages without success (Brutsaert *et al.*, 1946; Schultz and White, 1948). In the present experiments 2-day-old chicks were inoculated intracerebrally with 0·03 ml. of a 1 in 100 suspension in saline of mouse brain infected with Columbia SK virus. The infected chicks appeared normal but virus was present in the brain and cord for 72 hours after inoculation and, on histological examination of the cord, focal lesions, consisting of small nodules of infiltrating cells, were found in the grey matter. These nodules (fig. 6) were very similar to those seen in mice. When chickens were similarly inoculated with Lansing poliomyelitis virus no lesions could be seen as a result of the action of the virus.

Pigeons also were inoculated intraperitoneally with 0·1 ml. of a 1 in 100 suspension of mouse brain infected with Columbia SK virus. Pigeons killed 72 and 120 hours later failed to show virus in the blood or brain when these tissues were inoculated intraperitoneally into mice.

### *Experiments on survival in cockroaches*

The possibility that cockroaches may play some part in transmitting Columbia SK virus was studied. Hurlbut (1950) found that when Lansing virus was inoculated into the hæmatocele of the large-winged American cockroach *Periplaneta americana* and the whole cockroach was then triturated and injected into mice, the poliomyelitis virus survived for 15 days. Virus was not present in the fæces or in the eggs. Syverton and Fischer (1950) showed that, when *P. americana* was fed with 0·2 ml. of 10 per cent. mouse-brain suspension infected with spontaneous mouse-encephalomyelitis virus (Theiler), the virus could be recovered from the fæces from the first to the sixth day.

Experiments were carried out with Columbia SK virus and *Blattella germanica* and *P. americana.* The former were obtained from the Midlands, the latter from the forest region of tropical Africa, through the kindness of Dr O. Ampofo, Mampong Akwapim, Gold Coast. A 10 per cent. suspension of mouse brain infected with Columbia SK virus was injected into the hæmatocele sac of both species of cockroach, 0·1 ml. in the case of *B. germanica* and 0·2 ml. in the case of *P. americana.* Cockroaches were killed after 72 hours and 120 hours. The legs and wings having been removed the bodies were triturated in physiological saline containing 500 units penicillin and 100 μg. streptomycin per ml., centrifuged at *700 r.p.m.* for 10 minutes and the supernatant inoculated in a dose of 0·1 ml. intraperitoneally into mice. Virus was detectable 72 or 120 hours after inoculation in the bodies of *P. americana* but not in those of *B. germanica*.

Feeding experiments also were undertaken. A 10 per cent. saline suspension of mouse brain infected with Columbia SK virus was thoroughly mixed with bran and put in a small glass feeding-dish into the cage containing the cockroaches. Twenty-four hours later the cockroaches were removed and put into a clean cage. Their fæces were ground up, suspended in broth, and a Seitz filtrate of the suspension injected intraperitoneally into mice. Virus was present in the fæces of *P. americana* but not of *B. germanica* up to 8 days after the original feed, the longest period tested. It seems probable, therefore, that it was being excreted in the fæces. However, there exists another possibility; the bodies of the cockroaches, during their original period of feeding, may have become contaminated with Columbia SK virus; this virus may then have indirectly contaminated the fæces. Further experiments are being undertaken to determine how far such contamination is possible.

## DISCUSSION

The observations here recorded show that Columbia SK virus has a wide range of infectivity; what, to adapt a term from antibiotic chemotherapy, may be referred to as its "infectivity spectrum" includes cynomolgus monkeys, hedgehogs, cotton rats, mice, hamsters, guinea-pigs, the field vole, dormouse, and chickens. On the other hand, the Lansing strain of poliomyelitis virus has a narrower infectivity spectrum, restricted to monkeys, cotton rats and mice. There are also certain differences in the pathological changes produced especially in the type of nerve lesion; for the Columbia SK virus, although it attacks the anterior horn cells in the cord, produces very diffuse lesions. Both Columbia SK and the Lansing poliomyelitis virus can attack the heart muscle, though this tendency appears to be more pronounced with Columbia SK than with the Lansing virus.

Additional evidence to show the inter-relationship of the Columbia SK virus to Mengo encephalomyelitis virus is the fact that a Mengo encephalomyelitis antiserum neutralises Columbia SK virus after passage through hedgehogs whereas a Lansing antiserum has no such action.

## SUMMARY

1. Columbia SK virus produced a fatal infection of the hedgehog *Erinaceus europaeus* when inoculated by a variety of routes.

2. The virus was present in the blood and organs of hedgehogs but not in the urine or fæces.

3. The hedgehog flea *Archaeopsylla erinacei* took up Columbia SK virus from the blood of the hedgehog but did not transmit infection by its bite. When the flea was crushed and rubbed on the intact skin,

infection resulted. The virus did not survive in the cockroach *Blattella germanica* but did survive in the bodies of *Periplaneta americana* for at least 120 hours after injection, and possibly in the fæces for as long as 8 days after feeding.

4. Columbia SK virus was infective for the Orkney vole, *Microtus orcadensis*. Lansing poliomyelitis virus was not infective for this species by intracerebral or by other routes of injection.
5. Columbia SK virus after intracerebral injection produced an inapparent infection in the white rat. The virus was recovered from the brain after 13 but not after 20 days. No lesions were found in the central nervous system.
6. Columbia SK virus produced lesions in the cord of the 5-day chick but not in the cord of the pigeon.
7. Mengo encephalomyelitis immune serum neutralised Columbia SK virus after passage through the hedgehog.

Our thanks are due to Dr C. H. Andrewes, F.R.S., for the strain of Columbia SK virus, to Dr G. W. A. Dick for the Mengo encephalomyelitis immune serum, and to Professor G. A. H. Buttle, in whose laboratory these investigations were carried out. We also owe a debt of gratitude to Dr L. P. Clarke and his technicians for preparing the sections and to Mr F. Grigg for taking the photomicrographs. One of us (G. M. F.) is in receipt of a grant from the Medical Research Council.

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# OBSERVATIONS ON THE RELATIONSHIP OF SYMBIOTIC AND LYTIC BACTERIOPHAGE

J S K Boyd

*From the Wellcome Laboratories of Tropical Medicine, London*

(Plate LXXVII)

Symbiotic bacteriophage is present in every organism of an infected culture, and normally multiplies in unison with the host bacterium without causing apparent damage or bringing about lysis A limited number of free particles of active lytic phage are, however, to be found in association with such cultures and it is these which have given rise to the designation "lysogenic". Lwoff and Gutmann (1950) have shown by an ingenious technique that these free particles take origin from a few bacteria in the culture in which the balance between phage and bacteria is disturbed In these the normally inactive symbiotic phage assumes a lytic character and, having multiplied, brings about the disintegration of its bacterial host with an accompanying liberation of active particles into the surrounding medium. These free particles can be detected by their action on a sensitive strain of the same species of organism which serves in this respect as an "indicator"

It has been found (Boyd, 1950) that in the case of certain symbiotic phages of *Salmonella typhi-murium*, the free particles of phage which are present in infected bacterial cultures are capable of producing in organisms of the indicator strain either *(a)* a permanent symbiotic infection similar to that of the culture from which the particles were derived, or *(b)* a lytic cycle of development with the production of large quantities of free particles Little is known of the conditions which determine whether infection shall be symbiotic or lytic in nature, although it has been suggested that resistance of the bacterium plays a part by arresting the development of the phage (Lwoff and Gutmann) During an investigation of the growth curves of cultures infected with a phage of this kind a very curious phenomenon was observed which ultimately threw light on this problem and at the same time gave evidence of a type of variation hitherto undescribed in phage.

This paper gives an account of the phenomenon and of the various experiments which led to its elucidation, and discusses the conclusions which can be drawn therefrom.

### *Materials and methods*

Materials and methods are as detailed in a previous paper (Boyd). The indicator strains were *S. typhi-murium* 1404 and 1411; the phages were those of the A group, A1 being used as a routine and the others for confirmation.

The phage concentrates used in the experiments were made in one of two ways—by inoculating a broth medium either with both the indicator strain and the phage-infected strain or, alternatively, with the indicator strain and a loopful of a previous batch of concentrate. After overnight incubation the turbid cultures were cleared and freed from living bacteria by filtration through a Chamberland L3 filter, or by heating to 60° C. for 30 minutes and spinning. An accurate count of the number of phage particles was then made. By both methods a clear "lysate" containing approximately $10^{10}$ phage particles per c.c. was obtained.

## EXPERIMENTAL FINDINGS

### *Growth curves of* S. typhi-murium *when exposed to different concentrations of phage A*1

For purposes of comparison the "normal" growth curves of *S. typhi-murium* in the presence of different concentrations of a permanently lytic phage—one of the "O" phages—must first be described. In the experiment selected, phage was added to a series of tubes of broth to give concentrations of particles per c.c. of $3\times10^9$, $3\times10^8$, $3\times10^7$ and $3\times10^5$ and to each tube was added sufficient of a thick suspension of *S. typhi-murium* 1404 (the indicator strain) to give $1\times10^8$ bacteria per c.c. The ratio of phage to bacteria was, therefore, 30 to 1, 3 to 1, 3 to 10 and 3 to 1000. A control containing only bacteria was added to the series and the tubes were incubated in a water bath at 37° C. Absorptiometer readings were taken before incubation and at half-hourly intervals thereafter. The results were plotted and are shown in fig. 1.

In cultures where there are more phage particles than bacteria, lysis occurs without further bacterial multiplication. When the bacteria are more numerous than the phage particles, both multiply, the latter more rapidly than the former and lysis occurs when phage concentration overtakes bacterial concentration. In all cases a secondary growth of phage-resistant bacteria appears after about six hours' incubation. These results are in conformity with expectations.

When this experiment is repeated with a concentrate of phage A1 prepared as described and an indicator strain of *S. typhi-murium*, the curves follow a very different course.

No lysis is apparent when the quantities used are such that at the outset there are more phage particles than bacteria. The growth

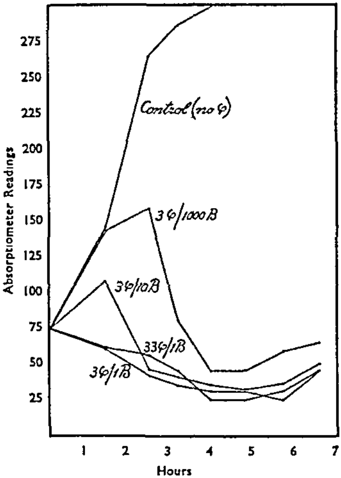

FIG 1 —Growth curves of *Salmonella typhi-murium* in the presence of a lytic "O" bacteriophage φ = phage particles B = bacteria.

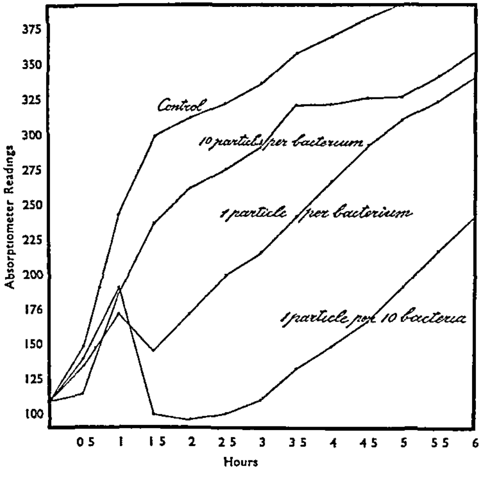

FIG. 2.—Growth curves of *Salmonella typhi-murium* in the presence of symbiotic bacteriophage A1.

## PLATE LXXVII

Figs. 3 to 6 show colonies of *Salmonella typhi-murium* which have, while growing, become contaminated with phage A1. All are ×4.

FIG. 3.—Contaminated at an early stage in its growth. Shows characteristic radial markings.

FIG. 4.—Colonies showing a small patch of central "necrosis" are contaminated at a slightly later stage than those showing only radial markings.

FIG. 5.—Contaminated at a still later stage. Well-marked patch of central necrosis. Note that these colonies are smaller than the others—a constant feature when these changes are present.

FIG. 6.—Contaminated at a late stage. A blemish appears on the margin of the colony and spreads round the circumference. Subsequently the centre of the colony becomes semi-translucent (presumably due to lysis) and the margin becomes overgrown with symbiotically infected organisms.

FIG. 7.—Plaques produced by the phage particles present in the supernatant fluid of a spun culture of *S. typhi-murium* symbiotically infected with phage A1. Note the great variation in size. The central disc in the plaques consists of phage-infected resistant bacteria.

FIG. 8.—Plaques produced by the phage particles present in a concentrate prepared from a culture in which phage A1 has multiplied by the lytic cycle in the indicator strain of *S. typhi-murium* 1404. Note the cluster of large and mature plaques of uniform appearance and one small immature plaque.

SYMBIOTIC AND LYTIC BACTERIOPHAGE

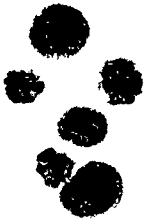

FIG. 3.

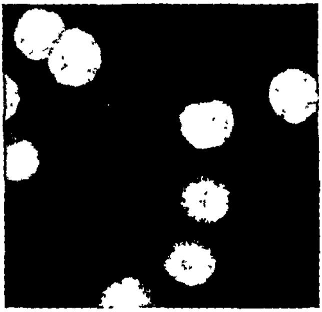

FIG. 4.

FIG. 5.

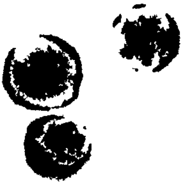

FIG 6

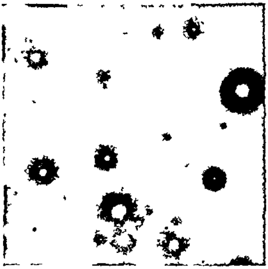

FIG. 7.

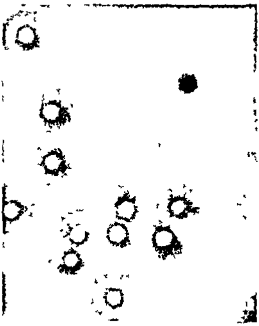

FIG. 8.

For the interpretation of these findings, the possible fate of the normal bacteria deposited on the surface of the phage-impregnated agar must be considered. One of three things may happen to each organism :—

(*a*) It may alight on a portion of agar where it is not in immediate proximity to a phage particle. On incubation it will multiply and form a colony. This may grow to full size without encountering phage, *in which case it will be normal in appearance. Alternatively, it may,* as it grows, come in contact with a particle, when phage action will spread through the colony, producing characteristic changes. It is proposed to designate such colonies "phage-contaminated" to distinguish them from the normal-looking colonies formed by the growth of a single symbiotically infected organism which will be referred to as "phage-infected" colonies.

The changes found in phage-contaminated colonies are illustrated in figs. 3 to 6. The different appearances are closely related to the stage of development of the colony when it comes in contact with the phage particle. If this takes place early, the 24-hour colony shows only radial markings seen in figs. 3 and 4. When invasion takes place at a later stage a small area of central "necrosis" is seen, as in figs. 4 and 5. Such colonies tend to be smaller than any of the others. When the colony encounters the phage at a late stage in its development, a marginal blemish appears which spreads round the circumference and finally gives the characteristic appearances seen in fig. 6. It is significant that if organisms taken direct from these different colonies are suspended in saline, diluted, and plated on agar so as to give discrete colonies, only symbiotically infected colonies develop from the first two types, whereas from the third type there is a mixture of contaminated and infected colonies.

(*b*) The organism may come directly in contact with a phage particle and be infected in such a way that the lytic cycle of development is initiated. In this case it will be destroyed and no colony will develop.

(*c*) It may come directly in contact with a particle which enters into symbiosis with it. It will then develop into a colony which is normal in appearance but is formed of phage-infected bacteria.

When the figures in table I are examined in the light of these considerations, the following conclusions can be drawn. At a concentration of $10^2$ particles per c.c. the phage has failed to reach any of the colonies and all are normal, while at $10^3$ it has contaminated about half. At concentrations of $10^4$, $10^5$ and $10^6$ all visible colonies have been contaminated and at $10^6$ some lysis has taken place. It is remarkable that in the majority of colonies exposed to the $10^6$ concentrations, the markings were of the radial type (figs. 3 and 4), while in the $10^5$ concentration they were of the small central type (figs. 4 and 5) and in $10^4$ of the large central type (fig. 6).

In the $10^7$ to $10^9$ concentrations a few contaminated colonies are present and presumably indicate that the mixing of phage through the medium has not been uniform. The majority of the colonies are normal in appearance and are symbiotically infected. The striking feature is the decrease in the number of such colonies as the phage concentration falls. This decrease, by inference, must be balanced by an increase in lytic infections. If the average number of organisms deposited on each plate in this experiment is taken as 130, the fate of the bacteria against the four highest concentrations is as recorded in table II.

TABLE II

*Fate of bacteria on phage-impregnated plates*

| Concentration of phage particles . . | $10^9$ | $10^8$ | $10^7$ | $10^6$ |
|---|---|---|---|---|
| Symbiotically infected . . . . | 87 | 42 | 33 | 0 |
| Lysed or phage-contaminated . . | 43 | 88 | 97 | 130 |

*Note.*—The figures in the bottom row are based on the assumption that each plate was inoculated with an average of 130 organisms.

The development of symbiotic infection is thus greatest when the organism is exposed to high concentrations of phage and is of a low order when the concentration is weak.

### *Development of symbiotic infection in broth cultures*

To confirm that the same type of action takes place in a fluid medium, a set of tubes was prepared similar to that used in investigating the growth curves. A standard number of organisms was added to decreasing decimal dilutions of phage in broth, the tubes were incubated for 30 minutes to allow fixation to take place, and then cooled in ice to arrest development while the various dilutions were prepared. Each culture was diluted to $10^{-6}$ so that approximately 100 organisms were present per c.c., and 0·5 c.c. of this was plated on agar and incubated to determine the condition of the organisms. At the same time the number of " infective centres " in each culture was estimated. Infective centres are counted by the same technique as is used for counting free phage particles. Each infective centre arises from either a free phage particle or from a bacterium to which phage particles have become adsorbed. Only one infective centre can take origin from a bacterium, irrespective of the number of phage particles that have invaded or have become attached to it. Here again, many experiments have given similar results. A typical set of figures is shown in table III.

In the higher phage concentrations there is a reduction in the number of colonies and it may be assumed that the others have failed to develop because of lytic infection of the parent organism. In a decreasing ratio, corresponding with the phage concentration, a proportion of the colonies are phage-infected. In the lower concentrations there are insufficient phage particles to ensure a significant number of contacts between bacteria and phage. In parallel with phage infection there is a reduction in the number of infective centres, indicating that in some cases several phage particles have become adsorbed on to one bacterium.

TABLE III

*Relationship of bacteriophage concentration to the development of symbiosis*

Salmonella typhi-murium *and bacteriophage A*1

| Phage particles per c.c.<br>Ratio of phage particles to bacteria | $1\cdot5\times10^{9}$<br>15/1 | $1\cdot5\times10^{8}$<br>1·5/1 | $1\cdot5\times10^{7}$<br>1·5/10 | $1\cdot5\times10^{6}$<br>1·5/100 | $1\cdot5\times10^{5}$<br>1·5/1000 | Nil<br>0/1 |
|---|---|---|---|---|---|---|
| Colony count . . | 28 | 22 | 41 | 39 | 45 | 58 |
| Phage-free colonies . | 1 | 14 | 40 | ... | ... | ... |
| Phage-contaminated colonies | 0 | 3 | 1 | ... | ... | ... |
| Symbiotically infected colonies | 27 | 5 | 0 | ... | ... | ... |
| Infective centres . . | $1\cdot7\times10^{8}$ | $4\cdot7\times10^{7}$ | $1\cdot2\times10^{7}$ | $1\cdot5\times10^{6}$ | $1\cdot5\times10^{5}$ | ... |

These results are very similar to those given by the experiment with phage-impregnated agar and confirm the observation that significant numbers of symbiotic infections occur only where the bacteria are exposed to the action of relatively high concentrations of phage.

### *Differences in plaque characters in symbiotic and lytic cultures*

When an overnight broth culture of symbiotically infected organisms is heated to 60° C. for 30 minutes and then spun, the supernatant contains a low concentration of phage particles which can be detected by spreading a drop on an agar plate previously sown with the indicator strain. The plaques which develop after incubation vary greatly in size and hence in stage of development. A number are mature, others are pin-point size and, between these extremes, all gradations occur. This finding is constant. Fig. 7 is from a plate prepared in this way.

In contrast to this, the plaques formed by the concentrate used in studying the growth curves and other experiments are very uniform in size and appearance. Nevertheless, when a plate is prepared in such a way as to show a large number of discrete plaques, a few are found to be of the small type. In one experiment, where a count was made, there were approximately 550 mature plaques, 10 small ones and some intermediate size ones, difficult to assess. There were, in all, from 5 to 10 per cent. of immature plaques. Fig. 8 is selected from a heavily infected plate to show the general uniformity in size, and one small plaque.

The significance of these findings will be discussed in due course.

## DISCUSSION

The results of these experiments furnish an explanation of the paradoxical growth curves in figs. 1 and 2. Whereas in the experiment with lytic O phage an increased concentration leads to earlier lysis, in the other it brings about a higher incidence of symbiotic infection. Organisms symbiotically infected are not destroyed and are resistant to further phage action. They multiply in normal fashion and thus give a steady increase in culture opacity. In fig. 2 the lag between the curve of the control and that of the highest phage concentration results from the lytic infection and subsequent disappearance of more than half the original inoculum of bacteria. In the lower concentrations of phage the curves show the effect of less symbiotic and greater lytic infection. Many bacteria are at first uninfected and so multiply until overtaken by the rapidly propagating lytic phage. It is noteworthy that in the case of A phage (fig. 2) the secondary growth appears at an earlier stage than it does in the case of O phage (fig. 1). This is because even in the lowest phage concentrations the symbiotically infected organisms in the one greatly outnumber the resistant variants in the other.

These findings dispose of one problem only to pose another. Why are symbiotic infections commoner when bacteria are exposed to a high concentration of A phage particles ? There appear to be two possible explanations.

The first is that symbiosis occurs when a bacterium is invaded by more than one particle of phage. It can be seen from table III that, in high concentrations of phage, multiple adsorption occurs but this does not necessarily imply multiple penetration. It is difficult to understand why the presence of more than one particle should lead to symbiosis nor can any precedent or analogous occurrence be recalled. In the absence of any positive evidence this theory must be regarded as improbable.

The second is that the particles produced during the lytic cycle of development are of two kinds, one a lytic particle capable of reproducing only the lytic cycle of development, the other a symbiotic particle endowed with the attributes necessary for establishing symbiosis. To fit in with the findings recorded, it is necessary to postulate that the lytic particles are much more numerous than the symbiotic ones and that the symbiotic particles are dominant and can establish themselves in the host organism to the exclusion of the lytic ones. Given such conditions, it can be seen that in a high concentration of phage, entailing a proportionate if lower concentration of symbiotic particles, a sufficient number of the latter will be present to infect a significant number of bacteria. In low concentrations of phage the symbiotic particles will be too few to have any appreciable effect and the picture will consequently be one of development by the lytic cycle, with the build-up of symbiotic particles revealing itself in the early onset of secondary growth.

Evidence of the existence of two types of particle is furnished by the experiments illustrated in figs. 7 and 8. The great majority of the plaques arising from particles produced by the lytic cycle of development (fig. 8) are large and uniform in size and appearance, though a few are small. It is justifiable to assume that the large plaques result from the immediate action of a lytic particle on the organisms of the indicator strain with which the plate is sown. In contrast, plaques produced by the particles present in the supernatant fluid of a spun culture of symbiotically infected organisms (fig. 7) occur in all stages of development. Of these the mature plaques no doubt spring directly from lytic particles. The smaller and younger plaques could well be explained as a later development from symbiotic particles which would, to begin with, produce symbiotic infection of the indicator organisms. In multiplying, these would, after a varying number of divisions, produce lytic variants just as they do in broth cultures, and such lytic particles, on meeting the adjoining normal and sensitive organisms, would give rise to plaques whose stage of development would depend on the time which had elapsed before the lytic particle was thrown off.

If then, the large uniform plaques are from lytic particles and the smaller ones from symbiotic particles, it would appear that, in the sporadic lysis which occurs during the growth of a symbiotically infected culture, symbiotic and lytic particles are produced in about equal numbers. Conversely, in the mass lytic multiplication occurring in the indicator strain, lytic particles outnumber symbiotic by about 20 to 1. The picture thus revealed is that of a two-way or diphasic variation, lytic particles being produced as mutants by the symbiotic phase and symbiotic mutants emerging during the lytic cycle of development.

This concept of two cycles of development is in general agreement with that of Lwoff and Gutmann, but these workers explain their findings in a different way. They postulate only one type of particle but believe that some bacteria are sensitive to its action while others possess certain powers of resistance. When a phage particle penetrates one of the latter its development is arrested and it remains in an inactive or latent condition. They regard phage in this state as "probacteriophage" and believe that it divides along with the infected bacteria. In certain circumstances this immature phage is "reactivated", when it resumes development and produces disintegration of the host bacterium with the liberation of active particles. These may either give rise to lysis in susceptible organisms with which they come in contact or may establish themselves in a state of latency in those which are resistant. These observations were made on *B. megatherium.*

As far as *S. typhi-murium* and phage A are concerned, the theory of bacterial resistance as a cause of "lysogenicity" is untenable. If this accounted for the development of symbiotic infection, then in a series of tests in which the same total number of bacteria is exposed to an adequate concentration of phage, the numbers which are either symbiotically infected and survive or lytically infected and destroyed, would be constant, irrespective of variations in the phage concentration. The figures in the tables show that this is not so. The numbers vary considerably, a high rate of symbiotic infection with a low rate of lytic infection being associated with a high phage concentration, and vice versa. There is nothing to suggest that bacterial resistance plays any part in producing symbiosis.

The theory that latent or symbiotic phage is immature lytic phage, though it has won considerable acceptance, nevertheless presents certain difficulties. It receives support from the observation that active particles are not liberated when symbiotically infected bacteria are artificially disintegrated in different ways (Burnet and McKie, 1929; Wollman and Wollman, 1936; Gratia, 1936). Recently, Lwoff, Siminovitch, and Kjeldgaard (1950) have induced mass lysis in a "lysogenic" culture by means of ultraviolet radiation, an observation which suggests that, under the influence of certain stimuli, development to maturity takes place. On the other hand, it is unusual for an immature body to multiply indefinitely, as must in this case occur when phage is to be found in every daughter cell taking origin from each infected organism.

The alternative suggestion now put forward is that symbiotic phage is a mature entity, which in its intracellular form is intimately associated with its host and cannot be dissociated from it by artificial means. It is destroyed when the host bacterium is destroyed and by agents which are inactive against it when it is in the free state. Its normal form of multiplication is in harmony with that of the host. It is, however, fundamentally unstable and from time to time throws off variants which develop after the manner of lytic phages, leading to the disruption of the host bacterium and the liberation of both symbiotic and lytic particles, each capable of invading and developing in characteristic fashion in sensitive bacteria. The latter initiate lytic development but here too, the instability reveals itself in the emergence of a few variant symbiotic particles among the many lytic ones.

To illustrate this diphasic variation the diagram devised by Lwoff and Gutmann for the cycles of development of phage derived from lysogenic *B. megatherium* has been modified and is reproduced in fig. 9.

Variation of a simpler kind has already been recorded (Boyd). Two phages of the B group, symbiotic in *S. typhi-murium*, have been shown to produce permanently lytic variants, *i.e.* variants which show no tendency to revert to the symbiotic state. One of these has all the characters (antigenic and otherwise) of a recognised O type of Salmonella lytic phage.

In much recent work there is a tendency to focus attention on the lytic cycle of phage development and to regard the phenomenon of lysogenesis as a rather unimportant side-issue, probably due to "association between exogenous virus and incompletely susceptible bacteria" (Hershey and Bronfenbrenner, 1948). As far as *S. typhi-murium* and its associated phages are concerned, this is certainly a mistaken point of view and there is good reason to believe that it is equally so in relation to other bacteria and their phages. In fact, it is probable that symbiotic phage has an all-important role to play in maintaining continuity of existence among the lytic types. In experiments with cultures of bacteria and phage, in which there is an artificial concentration of both elements, the two are brought into such close proximity that adsorption and subsequent development occur freely. Under natural conditions, when bacteria and phage particles are relatively scanty, this is far from being the case, for the

MULTIPLICATION OF PHAGE A1

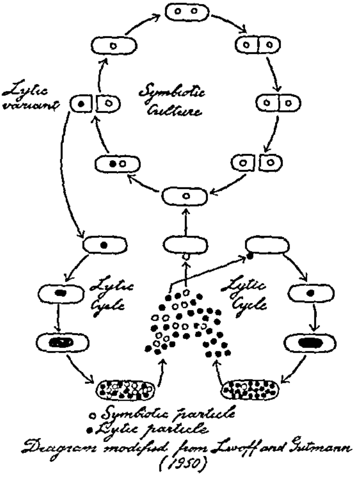

FIG. 9.—Diagram to illustrate diphasic phage variation Modified from Lwoff and Gutmann (1950).

distance which separates them may, in terms of their size, be enormous. In such circumstances the chances of a phage particle coming in contact with a potential host are limited and the likelihood of the phage maintaining itself in this way for any length of time is remote. Symbiotic phage, on the other hand, is firmly established in its host organism and has an assured life-span stretching to the death of the last bacterium of an infected strain.

It has long been known that phages which are symbiotic in one strain may be lytic in another—this was first recorded by Lisbonne and Carrère in 1922. Symbiotic strains as now described, endowed with the capacity of throwing off variants, are perhaps a more important source of lytic types and one which is unlikely to become exhausted. It is safe to predict that in time most lytic phages, which are now regarded as distinct entities, will be traced back to their symbiotic parents and that then the classification of phage will be based on symbiotic types and not, as at present, on ephemeral lytic variants.

If it be accepted that phages are in fact bacterial viruses, and there are now few who do not agree with this, it is pertinent to ask if plant and animal viruses have a similar life-history, with a latent and innocuous intracellular phase corresponding to symbiotic phage, and an active and pathogenic variant which is the counterpart of lytic phage. Such a conception is in broad agreement with facts already known regarding certain virus diseases and may, with wider application, provide a key to the solution of many obscure epidemiological problems.

## Summary and conclusions

1. When *Salmonella typhi-murium* is cultured in liquid medium in the presence of a high concentration of particles derived from group A phages which are normally found living in symbiosis with it, there is a slight lag in the growth curve followed by a steady increase in opacity. No obvious lysis occurs. When a similar culture is made in the presence of a low concentration of the same phage, there is a period of growth followed by abrupt lysis.

2. Experiments are described which show that this apparently paradoxical result arises from the fact that in the presence of a high concentration of A phage the majority of the bacteria become symbiotically infected and multiply without undergoing lysis. In low concentration only a few are symbiotically infected and phage development follows the lytic cycle.

3. The symbiotic infection which occurs in high concentrations of phage is considered to be due to the presence of two types of particle in the phage lysate used in the experiment—one of these, predominant numerically, is lytic and the other, which is scanty, has symbiotic properties.

4. It is concluded that phages of this type undergo a diphasic variation, the symbiotically infected bacteria producing lytic particles

from time to time, while symbiotic particles are produced during the lytic cycle of development.

5. This is of much significance, both in relation to the life-history of bacteriophage and as a pointer to the possible existence of analogous processes in plant and animal viruses.

My thanks are due to Mr R. A. Miles and Mr D. E. Bidwell for technical assistance in carrying out these experiments.

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(*Salmonella schwartzengrund*)

# AN OUTBREAK OF *SALMONELLA SCHWARTZENGRUND* INFECTION IN POULTRY

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*Salm. schwartzengrund* (I, IV, XII, XXVII . . . ; d⟷1, 7 . . .) was first isolated by Hohn from a case of enteritis in a human subject (Kauffmann, 1944). No further reports on its isolation have been found in the literature. The following is an account of *Salm. schwartzengrund* infection in poultry.

## METHODS

Material for examination was inoculated into selenite F medium (Leifson, 1936), the volume of medium depending on the nature of the material under examination. Incubation was carried out at 37° C. for 18-24 hrs. and the culture then sown on to Hynes's (1942) modification of Leifson's desoxycholate-citrate-agar with the addition of 1 per cent. sucrose and 1 per cent. salicin as described by Haines, Elliot, and Tomlinson (1947). The plates were incubated at 37° C. for 48 hours. Non-fermenting colonies resembling those of Salmonellæ were marked and tested for indole and urease production by the rapid test described by Smith and Chave (1949). Colonies negative to this test were subcultured and submitted to a slide agglutination test with polyvalent O and H antisera and, if necessary, with group-specific O and phase 1 and 2 antisera obtained from the Medical Research Council's Standards Laboratory, London. Organisms giving positive slide agglutination were submitted to a full tube test and their biochemical characteristics were determined.

The first three *Salm. schwartzengrund* cultures isolated in this outbreak were identified by Dr Joan Taylor. In the meantime, specific absorbed antisera were prepared for this purpose and were used for the identification of the remainder of the cultures.

## CLINICAL INVESTIGATIONS

*Salm. schwartzengrund* was first isolated from the liver of a six-day-old chick that had died suddenly. This chick was part of a unit of 25 chicks, and several of these units were kept in the same brooder house. As these chicks were to be used for experiments, efforts were made immediately to trace the extent and source of the infection. The brooder house was vermin- and bird-proof and was maintained under extremely good hygienic conditions. Composite fæces samples were collected from beneath each unit and inoculated into selenite medium. No Salmonellæ were isolated from any of these samples except those collected from the unit in which the chick had died. These contained *Salm. schwartzengrund.* In view of this, the remaining 24 chicks in that unit were killed and the intestinal contents of each was cultured. Surprisingly, all were negative for Salmonellæ.

It was considered possible that the chicks had been infected via the egg. Consequently, an examination was made of the adult hens, 20 in all, from which the particular unit of chicks was derived. Fæcal specimens from the hens were all negative for Salmonellæ, and later examination of individual sera from each hen did not reveal H or O antibodies for *Salm. schwartzengrund* in any of them. The small amounts of fæces expressed from the vent during sexing of day-old chicks were collected over a period of three weeks and cultured. No Salmonellæ were isolated from these specimens, which represented about 300 chicks; this also suggested that infection via the egg was improbable.

No further chicks were lost and it appeared that the death of the one chick was an isolated event. However, about 3 weeks later, unexpected losses occurred among groups of young chicks and turkey poults kept in two different animal houses as part of different experiments. Both houses were rat- and mouse-proof. The losses all occurred within a period of a few days and only occasionally with any signs of ill-health before death. *Salm. schwartzengrund* was isolated from the liver or fæces, or both, of 13 of the 18 dead chicks and seven of the eight turkey poults. Fæcal specimens were examined from *young chicks and turkey poults kept in other units; all were negative* for Salmonellæ. The infected units of turkey poults and chicks were supplied with food from the same bin and six samples of this dry mash were examined bacteriologically. Each sample consisted of about 2 oz. of dry mash. Two were found to contain *Salm. schwartzengrund.* The food material was carefully searched and a caked portion with a central core resembling rat fæces was tested and also found to contain *Salm. schwartzengrund.* The animal attendant mentioned that he had occasionally noted pellets of rat fæces in the food.

It now appeared that contaminated food was responsible for the outbreaks and that infected rats were probably the cause. The mash fed to the chicks consisted of a mixture of ingredients, some of which were grown on the premises and some of which were bought. An inspection of the granary revealed the presence of considerable amounts of rat and mouse fæces on the floor where the ingredients of the food were mixed. Some of the home-grown food, such as oats, was found to contain rat fæces. Specimens of the purchased food materials kept in sacks were cultured but none were shown to contain Salmonellæ. These included maize, maize meal, palm-kernel meal, bone meal, fish meal and meat meal. Samples of the mixed mash were examined at intervals afterwards but no more were found to contain Salmonellæ, showing that the contamination was sporadic.

A survey was now made to determine the extent of the infection on the premises with particular regard to wild rats and mice, which appeared to be the only animals, with the possible exception of sparrows, which might contaminate the home-grown food supplies.

### *Results of survey*

*Rats.* *Salm. schwartzengrund* was isolated from the fæces of two of ten wild rats that had been shot, and from three of 35 specimens of rat fæces also collected around the premises. No dead rats were ever discovered and the two rats with positive samples were in excellent bodily condition suggesting that they were symptomless fæcal excreters.

*Mice.* *Salm. schwartzengrund* was recovered from the fæces of one of 16 house mice caught in a cottage on the premises.

*Rooks.* One of ten rooks shot near the premises was found to be a fæcal excreter of *Salm. schwartzengrund.*

*Turkeys.* Three of 40 adult turkeys were found to be excreting *Salm. schwartzengrund* in their fæces. Rats had easy access to these turkeys. Of eggs laid by these turkeys, 40, which were either infertile or contained dead embryos, were shown not to contain Salmonellæ.

*House-sparrows.* The fæces of 100 house-sparrows trapped on the premises were found not to contain Salmonellæ.

*Pigs.* Of ten pigs one was shown to be excreting Salmonellæ in the fæces.

*Cows.* Fæcal specimens taken from 20 cows on the farm were negative for Salmonellæ.

*Horses.* No Salmonellæ were isolated from the fæces of nine horses.

*Cats.* Six cats were kept on the premises. Rectal swabs from each of these cats did not contain Salmonellæ.

*Ducks.* Of ten ducks one was found to be a fæcal excreter of Salmonellæ.

*Chickens.* Composite fæcal samples representing about 400 young chickens were all negative for Salmonellæ.

### *Further clinical investigations*

No further losses were noted among the poultry until two months had elapsed, when about 25 turkey poults, aged approximately four weeks, died during a period of about seven days. Eight of these poults were examined *post mortem.* *Salm. schwartzengrund* was cultured from the liver or fæces, or both, of five of them. These young turkeys were kept in an outdoor ark. Eleven arks containing young chickens, young ducks, and young turkeys were kept in the same area as the infected turkeys and had been fed from the same supply. None of the birds in these eleven arks was noted to be ill, and Salmonellæ were not isolated from pooled specimens of fæces collected from beneath each of the arks.

Since the infection rate of *Salm. schwartzengrund* in rats on the premises was high it was decided to determine whether the infection was present in rats in neighbouring districts. Consequently, with the co-operation of the County Pests Officer, 250 rats obtained from different districts in Huntingdonshire were examined bacteriologically. The rats had been killed with various poisons. A sample of fæces from each rat was cultured, and portions of the liver, spleen, and the mesenteric lymph glands from every five rats were pooled and cultured. Only one Salmonella culture was isolated—a culture of *Salm. typhimurium* from the fæces of one of the rats.

About six months after the last outbreak among the young turkeys, it was possible to examine bacteriologically 48 rats that had been killed on the premises with poison. The rats were examined in the same way as the 250 already described, and Salmonellæ were not isolated from any of them.

### *Post-mortem appearances*

No significant features were noted during post-mortem examination of the chicks, the only abnormality being a diffuse inflammation of the small intestine with congestion of the associated blood-vessels. This feature was also present in the turkey poults together with lesions in the liver similar to those commonly found in *Salm. typhimurium* infection in the mouse. These lesions were small triangular areas of necrosis, the base of the triangle being on the periphery with the apex directed towards the centre of the liver.

### *Bacteriological findings*

All 50 strains of *Salm. schwartzengrund* that were isolated resembled each other in their morphological, cultural, and biochemical characteristics. The organisms possessed the typical properties of the genus Salmonella. They produced acid and gas in glucose, maltose, mannitol, dulcitol, sorbitol, xylose, rhamnose and arabinose within 24 hours at 37° C. Lactose, sucrose, salicin and inositol were not fermented in 21 days. The methyl-red test was positive, the Voges-Proskauer test was negative, indole was not formed and the citrate-utilisation test was positive. Gelatin was not liquefied, hydrogen sulphide was produced and urease was not formed.

## DISCUSSION

The evidence supports the view that the infection of the young chicks and turkeys with *Salm. schwartzengrund* was caused by contamination of their food before or during the mixing process with excreta from rats or mice, or both. The negative results of the bacteriological examination of 250 rats from neighbouring areas suggested that the infection was confined to the rats on the premises. It would be difficult to decide whether rats, mice, rooks, or adult turkeys were permanent reservoirs on the premises and how the initial infection arose. The negative results of the bacteriological examination of 48 rats poisoned on the premises six months after the last outbreak of infection amongst the turkey poults suggested that the rats were not a permanent reservoir of infection although a definite conclusion could be drawn only if it had been possible to examine a greater number of rats.

It was obvious from the negative findings of the examination of fæcal specimens, carried out over a period of time from a large number of young and adult poultry, that the contamination of the food with *Salm. schwartzengrund* was a rare occurrence. Moreover, only portions of a contaminated batch of mixed food contained Salmonella organisms, and this was to be expected since the food consisted of dry mash which would probably allow neither multiplication nor even distribution of the bacteria.

## SUMMARY

An outbreak of *Salm. schwartzengrund* infection in young chicks and turkey poults is described.

Infection appeared to result from the contamination of food with the excreta of infected rats or mice.

*Salm. schwartzengrund* was isolated from the fæces of wild rats, adult turkeys, a mouse and a rook. Only two Salmonellæ were isolated from the fæces of 100 sparrows, a number of ducks, cats, cows, pigs and horses. All these animals were kept or caught on the infected premises. *Salm. schwartzengrund* was not isolated from any of 250 rats from neighbouring districts, but the fæces of one contained *Salm. typhi-murium.*

We wish to thank Mr K. A. Piggins and Miss Sheila Day for their technical assistance and Dr Joan Taylor for kindly identifying the first three cultures of *Salm. schwartzengrund.*

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# ACUTE HISTAMINE EROSIONS IN THE STOMACH AND DUODENUM OF GUINEA-PIGS

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(Plates LXXVIII-LXXX)

Repeated aqueous injections of histamine have in the past been given in an attempt to produce gastric erosions and ulcers in laboratory animals. Büchner and Molloy (1927) and Bürkle-de la Camp (1929) had some success with rats, but Overgaard (1931) and Orndorff *et al.* (1935), using dogs and O'Shaughnessy (1931) and Heinlein and Kastrup (1937-38), using cats, all reported negative findings.

Code and Varco (1940) first used beeswax as a base for the parenteral administration of histamine. Their method allows the gradual liberation of very large doses of histamine over 24 hours or more. By its adoption, Hay *et al.* (1942) have regularly produced chronic gastric and duodenal ulcers in many animal species including cats, dogs, guinea-pigs, rabbits and monkeys.

Less attention has been given to the earliest lesions in the stomach and duodenum after histamine injections. Olovson (1950), using a histamine-beeswax preparation in guinea-pigs, concluded that ulceration may be completed in less than 24 hours. His doses of histamine ranged from 0·1 to 10 mg. He noted, in some cases, mucosal hæmorrhages after half an hour and macroscopic erosions after 3 hours. He also observed that duodenal lesions greatly outnumbered gastric ones. No mention was made of control animals.

The following experiments represent an attempted confirmation of Olovson's work. Olovson removed the stomach and duodenum of his animals immediately after death and carefully washed the mucosa with running saline before examining it. Because of the possible risk of abrasion of the delicate mucosa, this method was modified by filling the stomach and duodenum *in situ* with 10 per cent. formol-saline with the aid of a small needle and syringe. About half-an-hour afterwards, stomach and duodenum were removed as one piece and immersed in a bath of 10 per cent. formol-saline before opening. Another modification of Olovson's method consisted in the use of 4·5 per cent. (w/v) white beeswax in arachis oil as base for the histamine. To each millilitre of base were added 5 mg. of histamine acid phosphate. Before injection, the histamine preparation was warmed to 50° C. for several minutes and shaken well.

## METHOD

Twenty-nine male guinea-pigs, whose average weight was 350 g., were injected intramuscularly with the histamine preparation immediately after the last meal of the day. The dose varied between 2 and 12 mg. histamine phosphate. They were killed within 24 hours, on the average after 15 hours, by a sharp blow on the head. The abdomen was opened immediately and the stomach and duodenum filled with formol-saline. Twenty male guinea-pigs of similar weight were used as controls. They were injected at the same time and in the same site as the test animals with 2 ml. of the arachis oil-beeswax base alone. They were killed after 15 hours by a blow on the head and their stomachs and duodenums were fixed in the same manner as those of the test animals.

## RESULTS

The incidence and distribution of the lesions are summarised in the table. The macroscopic appearances of the erosions are illustrated in fig. 1 (*a* and *b*), and the microscopic appearances in figs. 4 to 6 inclusive.

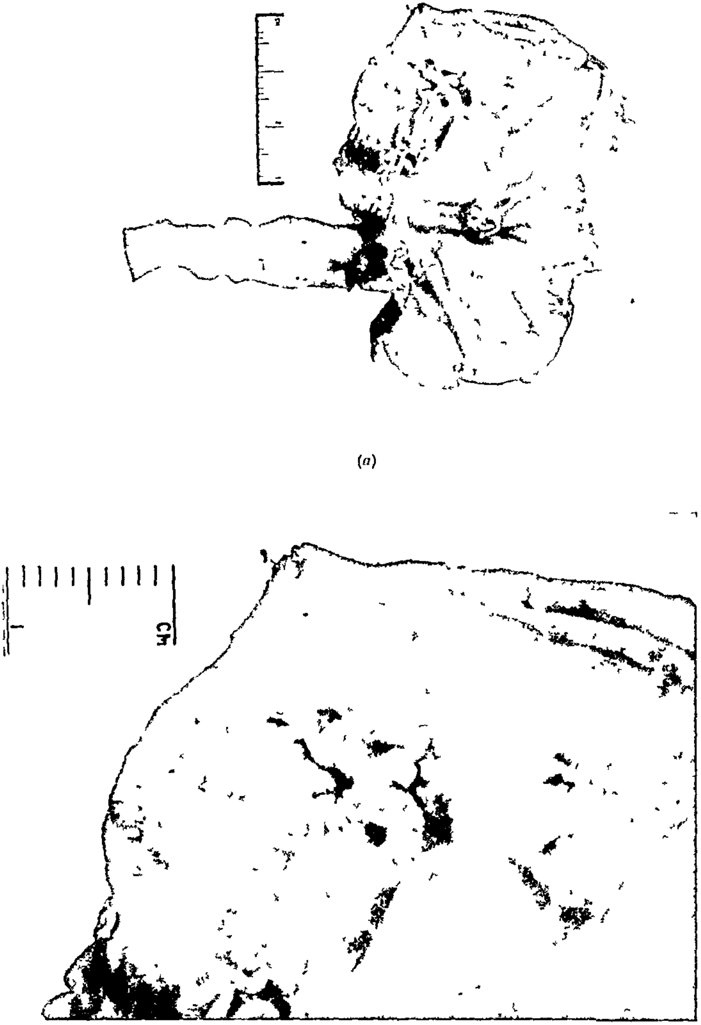
TABLE

*The incidence of stomach and duodenal lesions*

| Dose of histamine | No. of animals tested | No. of animals with stomach lesions only | No. of animals with duodenal lesions only | No. of animals with both stomach and duodenal lesions | No. of animals without stomach or duodenal lesions |
|---|---|---|---|---|---|
| EXPERIMENTAL ANIMALS | | | | | |
| 2·5 mg. | 3 | 2<br>(hæmorrhages only) | 0 | 0 | 1 |
| 5·0 mg. | 5 | 4<br>(erosions) | 0 | 0 | 1 |
| 7·0 mg. | 8 | 3<br>(2 with erosions; and one, which died 4 hours after injection, with hæmorrhages only) | 2<br>(erosions) | 2<br>(erosions) | 1 |
| 10·0 mg. | 9 | 4<br>(erosions, which in 2 animals had developed into perforations) | 1<br>(erosions) | 3<br>(erosions) | 1 |
| 12·0 mg. | 4 | 4<br>(erosions) | 0 | 0 | 0 |
| CONTROL ANIMALS | | | | | |
| 2 ml. of base only | 20 | 2<br>(erosions; a single, small one in one animal and 3 small ones in the other) | 0 | 0 | 18 |

Erosions were present in 22 of the 29 test animals, in the stomach in 14, in both stomach and duodenum in 5, and in the duodenum in 3. Mucosal hæmorrhages alone were present in the stomachs of 3 of the 29 animals.

HISTAMINE EROSIONS



(a)

(b)

FIG. 1.—(a) Non-hæmorrhagic acute erosions in the body of a guinea-pig stomach; (b) enlargement of part of (a).

With the smallest dose of histamine there were mucosal hæmorrhages only. With larger doses there were multiple erosions, hæmorrhagic or non-hæmorrhagic, and varying in size from a pin-head to 0·5 cm. in diameter. The erosions were round, oval or irregular in shape. They occurred in any part of the gastric mucosa but most commonly in the body or pyloric antrum. In the fresh state of the stomach they were clearly visible through the serosa (fig. 1). The duodenal erosions appeared as brown "scorched" spots or streaks, extending sometimes for 2 cm. from the pylorus and most prominent on the posterior wall.

Histologically, the erosions consisted of abrupt deficiencies in the mucosa. Often only the most superficial part of the mucosa was affected. At other times the underlying muscle was exposed. Evidence of hæmorrhage was frequently present in the necrotic floor, but inflammatory cells were, as a rule, conspicuous by their absence. The surrounding mucosa showed a remarkably healthy appearance. Occasionally, congestion and œdema of its superficial part were seen; more frequently, some degeneration and shrinking of glandular epithelium. Œdema of the submucosa was sometimes prominent. Some duodenal erosions were accompanied by a brisk inflammatory reaction, with numerous neutrophil polymorphs migrating into the tissue spaces, but, as has been stated, this reaction was exceptional.

The erosions were formed very quickly, *e.g.* in one animal injected with 10 mg. histamine and killed 4 hours later there were numerous, extensive gastric erosions, and in 2 animals gastric perforation occurred 5 and 8 hours respectively after the injection of 7 mg. histamine. An increase in the number, and sometimes of the size, of the gastric erosions accompanied increase in the dose of histamine. The optimum dosage for the production of erosions appeared to be between 7 and 10 mg. histamine. Duodenal erosions appeared only after administration of 7 or more mg. histamine.

There was no correlation between the state of the stomach and that of the duodenum. Some of the most extensively eroded stomachs were unaccompanied by duodenal lesions.

The presence of erosions in the stomachs of 2 of the control guinea-pigs was an unexpected finding. Presumably, acute erosions can occur spontaneously in guinea-pigs.

An irregular finding in the animals was that of scattered areas of acute liver necrosis. The necrotic areas measured 1 to 3 mm. in diameter and were more marked at the periphery of the organ.

## COMMENT

The results confirm Olovson's observations on the ease and speed with which acute erosions are produced with histamine injections. Unlike Olovson's results, however, gastric lesions greatly outnumbered duodenal ones.

The pathogenesis of the acute erosions presents a fascinating problem. Histamine is known to increase gastric secretion. Gastric juice destroys the mucosa once the surface epithelium has been disrupted. But, if the gastric juice were responsible also for the destruction of the surface epithelium of the stomach and duodenum, one would expect to find large areas of erosion following histamine injection, instead of which we find focal areas of erosion. Bishton's studies (1950) on the production of acute ischæmic lesions in the stomach and duodenum of guinea-pigs are possibly of significance in this respect. Following the parenteral administration of pilocarpine, he observed ischæmic lesions in the mucosa 20 minutes later. These were demonstrable by injecting the blood vessels with colloidal silver iodide and then exposing the tissues to a photographic developer. Bishton concluded that the lesions were due to vascular occlusion and were a forerunner of erosions.

It is possible that acute histamine erosions, as well as other types of erosions in the stomach and duodenum, are primarily vascular in origin. Their scattered distribution and the healthy surrounding mucosa are features pointing to a possible vascular basis.

Acute erosions, identical in histology to acute histamine erosions, are not uncommon agonal lesions in the human stomach and duodenum (Williams, 1950). Examples are illustrated in figs. 2 and 3. Similar lesions occur also during active life in a variety of conditions, such as acute emotional crises, and they have been noted in experimental animals following the administration of drugs such as pilocarpine, morphine and caffeine.

## SUMMARY

1. Acute erosions were produced in guinea-pigs by the intramuscular injection of 2-12 mg. histamine phosphate in a beeswax-arachis oil mixture.

2. Gastric erosions were far more frequent than duodenal erosions.

3. There was no constant correlation between the state of the stomach and that of the duodenum.

4. The erosions were formed very quickly, in some instances within 4 hours.

5. The character of the erosions suggests a primary ischæmic origin.

Thanks are extended to Professor W. D. Newcomb for his advice, to Mr Alec Beasley for histological preparations, to Mr Thomas Norris of the Wright-Fleming Institute and to the members of the Photographic Department, St Mary's Hospital, for their assistance.

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HISTAMINE EROSIONS

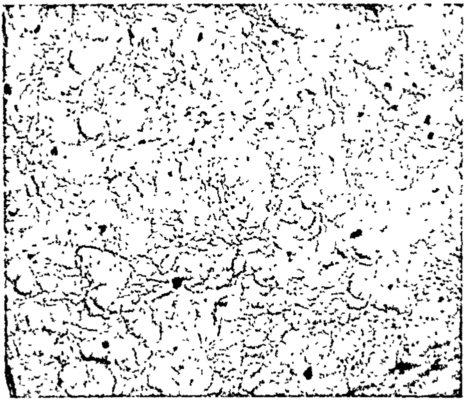
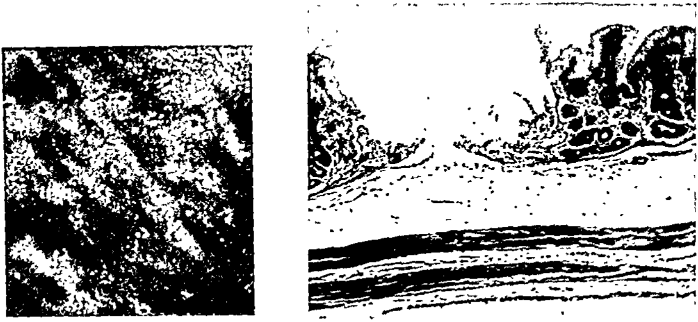


FIG. 2.—Hæmorrhagic acute erosions in the antral mucosa of a man, aged 87, who died of pulmonary embolism. The stomach was fixed with formol-saline immediately after death. × 1·5.



(a) (b)

FIG. 3.—(a) Multiple acute erosions in the antral mucosa of a premature male infant. × 2·5. (b) One of the erosions. (H. and E. × 90.) Note the absence of inflammation. The stomach was fixed with formol-saline immediately after death.

HISTAMINE EROSIONS

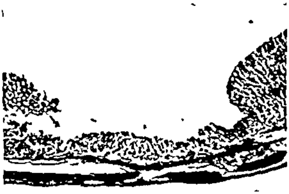


FIG 4.—Acute erosion in the stomach of a guinea-pig. There is no inflammation and the adjacent mucosa looks healthy. H. and E. ×40.

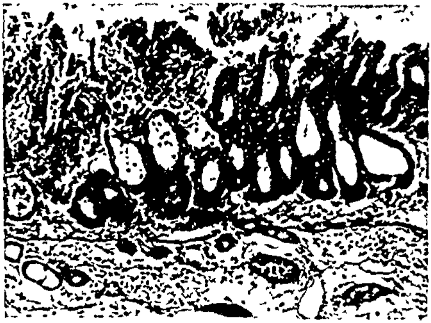
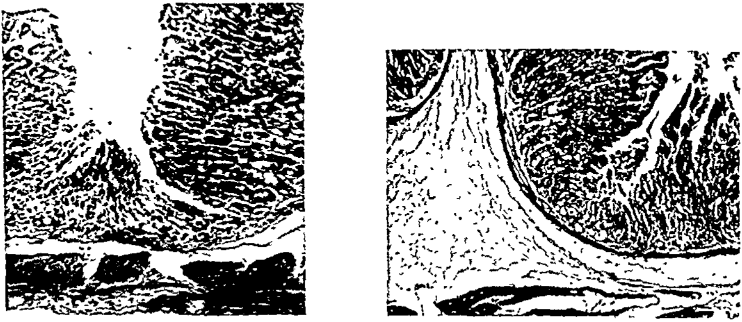


FIG. 5.—Acute duodenal erosions in a guinea-pig. The disintegration of the superficial part of the mucosa is apparent. Acute inflammatory changes are brisk. H. and E. ×120.



(a) (b)

FIG. 6 (a) and (b).—Small acute gastric erosions in a guinea-pig. In both sections the adjacent mucosa is healthy except for some tubular atrophy. Œdema of the submucosa is present in (b). H. and E. (a)×85; (b)×75.

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# QUANTITATIVE STUDIES ON THE CONTENT OF NUCLEIC ACIDS IN NORMAL AND LEUKÆMIC CELLS, FROM BLOOD AND BONE MARROW

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(Plate LXXXI)

Samples of aspirated human sternal marrow, venous blood, and leucocytes isolated from venous blood have been analysed for content of deoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP), and enumeration of nucleated cells in the samples has been carried out. The results are expressed in terms of average DNAP and RNAP in $\mu g. \times 10^{-7}$ per cell. A series of normal, healthy individuals has been studied, together with patients with various types of leukæmia, both before and during therapy. Such results express the averages per cell from the mixed populations of growing, differentiating cells and mature cells in marrow, and for the mixture of various types of mature leucocytes in normal blood, or leucocytes at varying developmental stages in the leukæmias.

The work of Boivin, Vendrely and Vendrely (1948), Vendrely and Vendrely (1949), and of Mirsky and Ris (1949) suggests a very constant content of deoxyribonucleic acid (DNA) for the individual somatic cell nuclei of any one vertebrate species; the haploid spermatozoa have approximately half this amount. Considerable differences are found between one species and another.

## Technique

*Bone marrow.* Samples were obtained by aspiration from the sternum, 0·2-0·3 ml. sufficing. Films for cytological examination were prepared, and the remainder of the sample mixed with 0·5 ml. of heparinised saline and weighed. From this mixture of known proportions, 0·1 ml. was withdrawn and diluted for enumeration of nucleated cells, and the residue used for determination of nucleic-acid content.

*Peripheral blood.* 2·5 ml. of freshly withdrawn venous blood were used for nucleic-acid determination, and 0·1 ml. diluted for nucleated cell count. Changes in the cells were minimal, but the samples were difficult to work with because of the great excess of protein.

In a number of cases, analyses were performed on isolated leucocytes. These cells could readily be obtained from leukæmic blood by heparinising and standing at room temperature for 1-1½ hours. The leucocyte suspension was then separated from the sedimented erythrocytes. With normal blood, a similar separation was achieved by the method of Minor and Burnett (1948), in which bovine fibrinogen is added to the fresh heparinised blood, and glassware is siliconed to minimise loss of polymorphonuclear leucocytes.* In some analyses, the leucocyte suspensions were freed from all adherent plasma proteins by washings with 0·85 per cent. sodium chloride solution. The leucocyte suspensions were then sampled for cell counting and analysis.

*Cell enumeration.* Dilutions of the marrow samples were made at 1 in 20 or 40 with 0·85 per cent. sodium chloride solution tinged with methyl violet, and cellular fragments broken up and the cells evenly suspended by slow mechanical rotation for 10 minutes. The improved Neubauer hæmocytometer chamber was used for counting. Where enumeration of nucleated cells only was required, the erythrocytes were lysed with saponin. Leucocyte suspensions were counted similarly, but where saline-washed suspensions were used, rapid mixing and counting was necessary to avoid clumping of cells.

The counts were performed on both sides of the chamber, 10 areas of 1 sq. mm. each being covered, usually giving a total of more than 1000 cells.

*Determination of nucleic acids.* The samples were extracted twice with cold 10 per cent. trichloracetic acid, and the nucleic acids determined, as described in an earlier publication on marrow (Davidson, Leslie and White, 1948), utilising the Schmidt and Thannhauser (1945) method for separating the acids, and determining them as inorganic phosphorus after perchloric-acid oxidation by the Davidson and Waymouth (1943) modification of Berenblum and Chain's (1938) micro-method.

*Cytological examination.* In addition to air-dried films stained by Romanowsky dyes, wet-fixed films were examined cytochemically by various methods for nucleic acids and proteins (see White, 1947, 1950).

## Results

### *Normal bone marrow*

The results for 27 aspiration biopsies of marrow from 22 normal volunteers are shown in table I and fig. 1. DNAP and RNAP is expressed in $\mu g. \times 10^{-7}$ per cell, and the ratio of the two acids, RNAP/DNAP, is given. The mean of observations is given for each parameter with the observed ranges and standard errors of the observations.

### *Leukæmic bone marrow*

The results for 33 observations on 17 cases of leukæmia of various types are shown in table I and fig. 2. These include observations made during various types of therapy, which in some cases produced remission (*vide infra*). The ranges are similar to those for the normal series. Seventeen initial observations on the 17 untreated patients give means which are similar to those for the total observations. Table III lists the types of leukæmia studied.

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* The leucocyte separations were carried out in co-operation with Dr J. W. Fawcett.

TABLE I.—*Nucleic acid content of normal and leukæmic blood and bone-marrow cells*

| | Values for nucleic acid phosphorus in μg ×10⁻⁷ per cell | | | Means for special groups within the series |
|---|---|---|---|---|
| | DNAP | RNAP | Ratio DNAP/RNAP | |
| *Normal marrow* | 27 observations on 22 individuals | 27 observations on 22 individuals | | Initial 22 observations on 22 individuals<br>DNAP 8·66 RNAP 7·05 Ratio 0·83 |
| Mean | 8·69 | 6·90 | 0·81 | |
| S E | 3·09 | 3·18 | 0·27 | |
| Observed range | 4·0-15·0 | 2·1-14·4 | 0·43-1·9 | |
| *Leukæmic marrow* | 33 observations on 17 patients | 31 observations on 16 patients | 33 observations on 16 patients | Initial observations on untreated patients<br>DNAP 8·26 (17 obs.) RNAP 7·10 (16 obs.) Ratio 0·90 (15 obs.) |
| Mean | 8·62 | 7·52 | 0·90 | |
| S E | 2·93 | 3·54 | 0·32 | |
| Observed range | 3·9-17·4 | 2·6-17·4 | 0·3-1·8 | |
| *Normal blood* | 11 observations on 10 individuals | 7 observations on 6 individuals | 7 observations on 6 individuals | Analyses of whole blood samples<br>DNAP 6·78 (6 obs.) RNAP 2·17 (2 obs.) Ratio 0·18 (2 obs.)<br>Analyses of 5 samples of isolated leucocytes<br>DNAP 7·34 RNAP 2·52 Ratio 0·38 |
| Mean | 7·04 | 2·42 | 0·35 | |
| S E. | 2·33 | 0·74 | 0·16 | |
| Observed range | 4·9-12·4 | 1·1-3·1 | 0·15-0·55 | |
| *Leukæmic blood* | 18 observations on 15 patients | 18 observations on 15 patients | 18 observations on 15 patients | Analyses of 12 whole-blood samples<br>DNAP 6·72 RNAP 3·75 Ratio 0·60<br>Analyses of 6 samples of isolated leucocytes<br>DNAP 7·55 RNAP 3·89 Ratio 0·48 |
| Mean | 6·99 | 3·8 | 0·56 | |
| S E. | 1·94 | 1·775 | 0·22 | |
| Observed range | 3·5-11·3 | 1·0-8·8 | 0·13-1·0 | |

### *Normal blood and isolated leucocytes*

In table I and fig. 1 the results are shown for a series of 11 observations on 10 normal individuals. The means for analyses on whole blood and on isolated leucocytes are for the combined results.

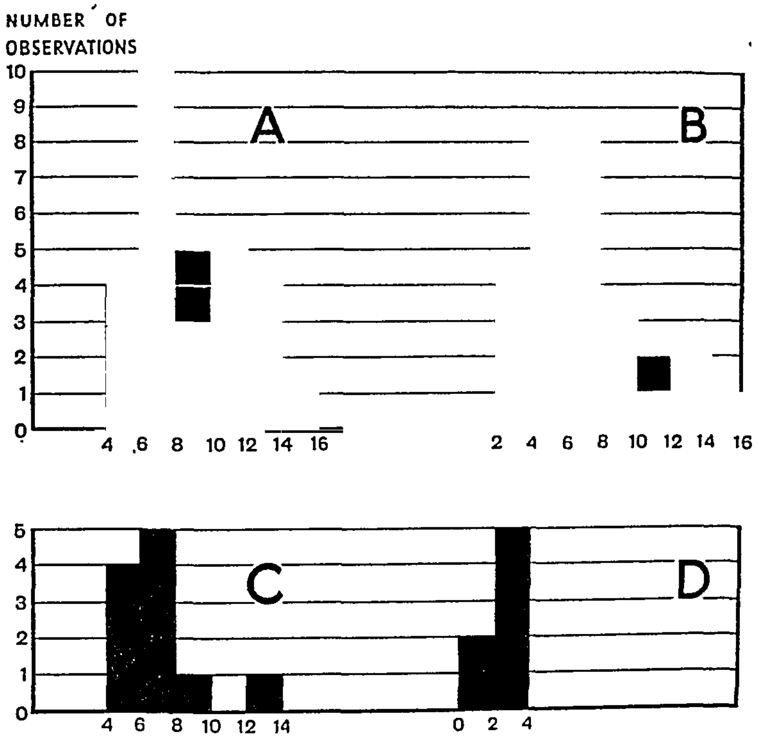

FIG. 1.—Average nucleic acid phosphorus (NAP) in $\mu$g. $\times 10^{-7}$ per cell in normal marrow and peripheral blood leucocytes.

Histograms

A. Deoxyribonucleic acid phosphorus (DNAP) in marrow cells. 27 observations on 22 individuals.

B. Ribonucleic acid phosphorus (RNAP) in marrow cells. 27 observations on 22 individuals.

C. DNAP in peripheral blood leucocytes. 11 observations on 10 individuals.

D. RNAP in peripheral blood leucocytes. 7 observations on 6 individuals.

### *Leukæmic blood*

Eighteen observations on 15 patients with various leukæmias are given in table I and fig. 2. Twelve observations were made on whole blood, and 6 on isolated leukæmic leucocytes. The types of leukæmia are listed in table III.

### *Comparison of results*

Student's " t " test (Fisher, 1950) has been used to compare the corresponding means of the different series. This is shown in table II, and the probability (P) is given where significant differences exist between means.

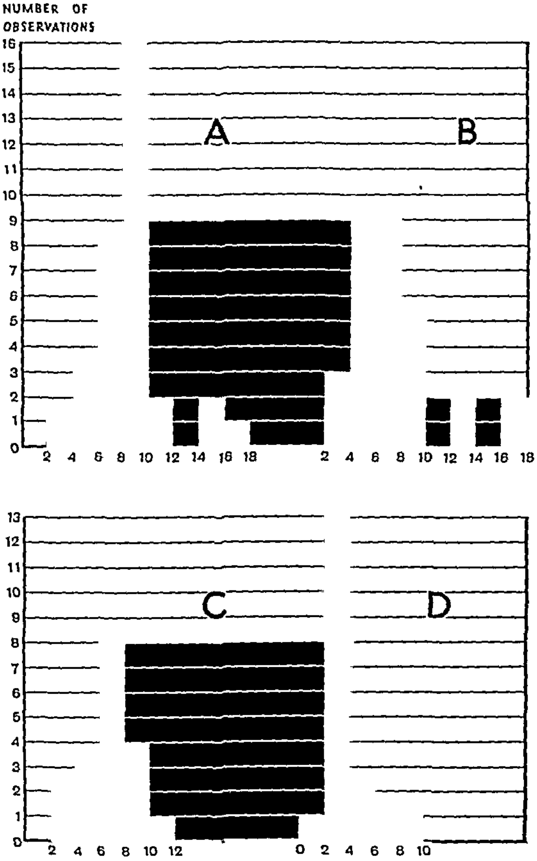

FIG. 2.—Average NAP in $\mu g. \times 10^{-7}$ per cell in leukæmic marrow and in peripheral blood leucocytes.

Histograms

A. DNAP in marrow cells. 33 observations on 17 patients.
B. RNAP in marrow cells. 32 observations on 16 patients.
C. DNAP in peripheral blood leucocytes. 18 observations on 15 patients.
D. RNAP in peripheral blood leucocytes. 18 observations on 15 patients.

### *Variation in the leukæmias*

The leukæmic patients are classified clinically and cytologically as far as possible into acute, subacute and chronic groups, characterised respectively by excess of primitive blast cells, varying proportions of primitive and older cells, and pronounced excess of the more differentiated cells. Expression of the nucleic acid content on this basis is shown in table IV, for 13 untreated cases..

TABLE II

*"t" test of significance for differences between means*

| Means compared | DNAP | RNAP | Ratio RNAP/DNAP |
|---|---|---|---|
| *Normal and leukæmic marrow* . | Not significant | Not significant | Not significant |
| *Normal marrow and normal blood leucocytes* | Doubtful significance | Highly significant | Highly significant |
| P . . . . . . | 0·05 | $<0·001$ | $<0·001$ |
| Degrees of freedom . . | 37 | 33 | 33 |
| *Leukæmic marrow and leukæmic blood* | Just significant | Highly significant | Highly significant |
| P . . . . . . | 0·02 | $<0·001$ | $<0·001$ |
| Degrees of freedom . . | 50 | 50 | 50 |
| *Normal and leukæmic bloods* . | Not significant | Significant | Significant |
| P . . . . . . | ... | 0·02-0·01 | 0·01 |
| Degrees of freedom . . | 28 | 24 | 24 |

TABLE III

*Types of leukæmia examined*

| Type of leukæmia | No. of patients in whom marrow was examined | No. of patients in whom blood leucocytes were examined |
|---|---|---|
| Acute myeloblastic and para-myeloblastic | 8 | 2 |
| Acute lymphoblastic . . . . . | 0 | 3 |
| Subacute myeloblastic and para-myeloblastic | 6 | 3 |
| Subacute lymphatic . . . . . | 1 | 1 |
| Chronic myeloid . . . . . | 1 | 3 |
| Chronic lymphatic . . . . . | 1 | 3 |
| Total . . . . . | 17 | 15 |

TABLE IV

*Nucleic acid phosphorus (NAP) in the marrow cells of different leukæmic groups (mean NAP in $\mu g \times 10^{-7}$ per cell)*

| Group | No. of patients | DNAP | RNAP | Ratio RNAP/DNAP |
|---|---|---|---|---|
| Acute leukæmia . . . . | 6 | 8·8 | 8·78 | 0·96 |
| Sub-acute leukæmia . . . | 5 | 7·98 | 6·12 | 0·79 |
| Chronic leukæmia . . . . | 2 | 8·25 | 4·68 | 0·60 |

There is an apparent trend towards highest RNAP and ratio values in the acute group, intermediate values in the subacute, and lowest values in the chronic cases. However, an analysis of variance does not confirm the significance of the trend for these observations (Fisher).

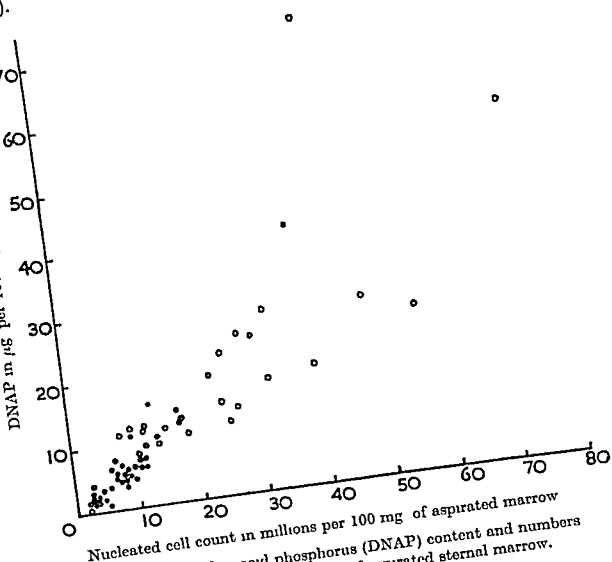

FIG 3.—Deoxyribonucleic acid phosphorus (DNAP) content and numbers of nucleated cells in unit weights of aspirated sternal marrow. Scatter Diagram

● Normal marrow (27 observations on 22 individuals)
○ Leukæmic marrow (32 observations on 16 patients)

### *Nucleated cell counts on aspirated sternal marrow*

Wide variation is found in the normal subjects (25,400-168,400 nucleated cells per mg. of aspirate). Variation is far greater in the leukæmias, which range from normal cellularity to gross hypercellularity (88,600-1,148,000), and with a mean significantly raised above normal ($P<0{\cdot}001$).

### *Nucleated cell count and DNAP content of aspirated marrow*

The scatter diagram, fig. 3, illustrates the relationship between nucleated-cell count and DNAP content per unit weight of aspirate. It is evident that their values in the leukæmias frequently exceed those in the normal range, but that considerable overlap occurs

### *Effect of therapy on leukæmic cells*

Where a response to therapy occurs, whatever the therapeutic agent or type of leukæmia, the general result is a decline in proportion of primitive blast cells and a rise in the more mature, differentiated cells in the marrow. Changes in the total cellularity of the aspirated marrow are very variable, however, particularly in the acute forms. No very regular changes have been found in the average nucleic acid content of the cells, but increased maturity of the marrow cells is often reflected by a fall in the average RNAP and in the RNAP/DNAP ratio. In some acute cases, however, the cells have possessed very little cytoplasm and the RNAP has been low before therapy, and an increase has occurred as the marrow becomes more mature.

The example of acute leukæmia summarised in table V illustrates changes occurring during a temporary remission induced by aminopterin.

TABLE V

*Aminopterin therapy in a patient with acute paramyeloblastic leukæmia*

| Date | Therapy | Marrow structure | Nucleated cells per mg. of aspirated marrow | Average NAP per cell in $\mu$g. $\times 10^{-7}$ | | Ratio RNAP/DNAP |
|---|---|---|---|---|---|---|
| | | | | DNAP | RNAP | |
| 14.6.49 | None | Predominance of paramyeloblasts | 170,800 | 8·9 | 11·6 | 1·3 |
| 20.6.49 | 3 days after 60 $\mu$g. of vitamin $B_{12}$ | Unchanged | 104,350 | 9·0 | 9·9 | 1·1 |
| 29.6.49 | After 9 mg. of aminopterin | Fewer blast cells, more mature cells | 100,600 | 8·3 | 6·8 | 0·82 |
| 13.7.49 | Blood | Mature, actively granulopoietic marrow | 245,300 | 9·4 | 8·1 | 0·86 |
| 1.9.49 | None | Blast cells again predominate | 181,700 | 6·4 | 7·0 | 1·1 |
| 22.9.49 | 18 mg. aminopterin from 3 to 11.9.49 | Blast cells predominate (post-mortem) | Cells not enumerated | ... | ... | 1·8 (Post-mortem) |

### *Influence of technique on the results*

An attempt was made to determine whether the suspension of marrow samples in heparinised 0·85 per cent. sodium chloride solution, as used in our technique, resulted in any loss of nucleic acids. Marrow samples from 9 normal individuals and 5 leukæmic patients were each divided and half transferred directly to 10 per cent. trichloracetic acid, half to 0·5 ml. of 0·85 per cent. sodium chloride solution in the usual way. The contents of nucleic acids in the paired samples were then compared on the basis of DNAP and RNAP per 100 mg. of aspirate and table VI shows the means of the amounts in the saline-treated samples as compared with means of the amounts in the samples taken direct into trichloracetic acid.

The apparent loss of RNAP in the saline treated samples as compared with samples treated directly with trichloracetic acid is not statistically significant, however, when an analysis of variance is applied to the figures (table VII)

TABLE VI

*Effect of suspension of marrow cells in 0 85 per cent sodium chloride solution prior to trichloracetic acid extraction*

| | | Mean nucleic acid phosphorus in µg per 100 mg of aspirate | | |
|---|---|---|---|---|
| | | Mean DNAP | Mean RNAP | Ratio RNAP/DNAP |
| 9 normal samples | (a) Aspirate immediately extracted with trichloracetic acid | 5 75 | 4 71 | 1 04 |
| | (b) Aspirate suspended in 0 85 per cent sodium chloride solution | 5 82 | 4 21 | 0 77 |
| | Percentage difference between (a) and (b) | +1 2 | −10 6 | |
| 5 leukæmic samples | (a) Aspirate immediately extracted with trichloracetic acid | 21 34 | 19 44 | 0 99 |
| | (b) Aspirate suspended in 0 85 per cent sodium chloride solution | 22 42 | 16 64 | 0 79 |
| | Percentage difference between (a) and (b) | +5 1 | −14 2 | |

Samples of aspirate have been divided into two portions (a) for immediate trichloracetic acid extraction, (b) for preliminary suspension in 0 85 per cent sodium chloride solution followed by extraction The mean nucleic acid phosphorus content of each group is expressed in µg per 100 mg of aspirate, and the percentage differences are expressed below

TABLE VII

*Nucleic acid phosphorus in aspirated marrow from 4 normal individuals*

| | | Normal marrow sample | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| (a) Whole aspirate | DNAP<br>RNAP<br>Ratio RNAP/DNAP | 1 9<br>1 1<br>0 58 | 3 6<br>1 6<br>0 43 | 4 9<br>3 5<br>0 71 | 11 1<br>10 9<br>0 98 |
| (b) Marrow fragments | DNAP<br>RNAP<br>Ratio RNAP/DNAP | 3 3<br>13 6<br>4 2 | 5 8<br>11 8<br>2 0 | 5 9<br>16 3<br>2 75 | 25 0<br>16 7<br>0 67 |
| (c) Blood separated from the fragments | DNAP<br>RNAP<br>Ratio RNAP/DNAP | 2 5<br>3 2<br>1 3 | 4 0<br>2 9<br>0 73 | 4 8<br>3 5<br>0 71 | 6 7<br>5 5<br>0 81 |

Each sample was divided into 3 fractions which were analysed separately — (a) whole aspirate, (b) cellular marrow fragments only, and (c) the blood separated by pipetting from the fragments NAP expressed as µg per 100 mg in each fraction

### *The nature of the aspirated marrow used for analysis*

Marrow aspirated from the sternum consists of cellular fragments suspended in blood. Preparation of histological sections from the aspirate shows that the fragments represent the true stromal, hæmopoietic tissue, whilst the adherent blood contains a mixture of mature peripheral leucocytes and more primitive cells liberated from the fragments.

In our earlier work (Davidson, Leslie and White), only the marrow fragments were analysed. The ratio RNAP/DNAP ranged from 3·8 to 1·0 in the normal series, with a value of 2·0 from the means for RNAP and DNAP. In the present series, the whole aspirate was used, and the results express average amounts of nucleic acid phosphorus for mixed populations of primitive and mature cells. This is responsible for the lower mean ratio RNAP/DNAP in the present normal series—0·81, with a range of 1·9 to 0·43.

Using sternal marrow aspirated from 4 normal individuals, an attempt was made to demonstrate this difference between the composition of whole aspirate and the cellular fragments. The fresh samples were divided at once into 3 fractions, which were analysed separately :—(*a*) whole aspirate, (*b*) the fragments of marrow remaining after removal with a pipette of the supernatant blood, this last being fraction (*c*), blood containing mature leucocytes and free marrow cells. The results are given in table VII.

The RNAP/DNAP ratios in the whole aspirate are much lower than the corresponding ratios for the fragments alone, and 3 of these are similar to the ratios previously found for fragments, but one is lower. An analysis of variance shows that the higher ratios for fragments are determined by their significantly higher content of RNAP, as compared weight for weight with whole aspirate ($P < 0{\cdot}001$).

## DISCUSSION

Although wide variation occurs between observations in both series, the means for the average DNAP per cell for normal and leukæmic marrow are very similar, as are the means for the cells in normal and leukæmic blood. A few figures for the DNAP content of other human cells are available (Davidson and Leslie, 1950). The present results are of the same general order as those obtained for cells of liver and kidney, although the results of Vendrely and Vendrely (1949) for liver are slightly lower than our values for hæmopoietic tissues. We have found half this amount of DNAP in human spermatozoa.

Métais and Mandel (1950) have studied the DNAP of human blood leucocytes by methods similar to ours, and their results show good agreement, both in the value of the mean and as regards the lack of any significant difference between normal and leukæmic leucocyte series ; our observed ranges and standard errors, however, are much

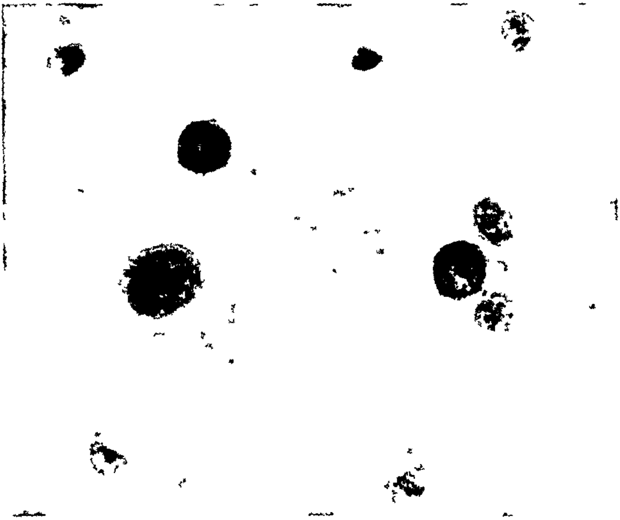

FIG 4 —Sternal marrow from a patient with acute paramyeloblastic leukæmia (see table V) Film wet fixed in Susa and stained by pyronin methyl green The more primitive cells have quite abundant deeply basophilic cytoplasm and large nucleoli Average DNAP per cell 8 9 μg $\times 10^{-7}$ RNAP per cell 11 6 μg $\times 10^{-7}$ Ratio RNAP/DNAP 1 3 ×900

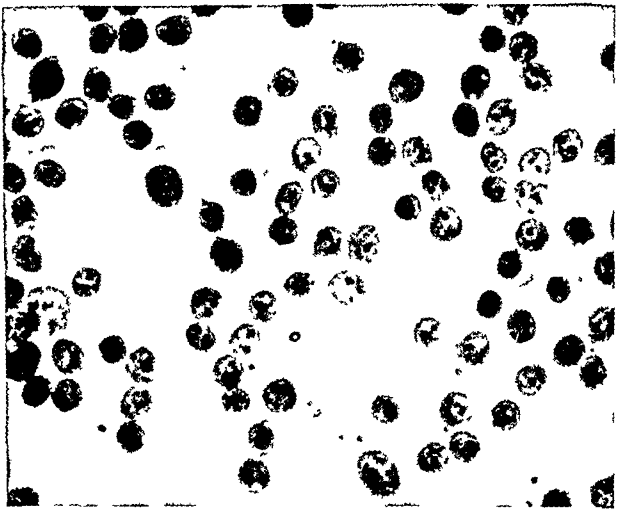

FIG 5 —Sternal marrow from a patient with acute myeloblastic leukæmia, prepared as in Fig 4 Most cells are small, with minimal cytoplasm, but possess prominent nucleoli Average DNAP per cell 8 7 μg $\times 10^{-7}$ RNAP per cell 4 3 μg $\times 10^{-7}$ Ratio RNAP/DNAP 0 49 ×900

larger than theirs. Osgood (1950, personal communication) has also studied the DNAP of normal and leukæmic human leucocytes, observing ranges similar to those encountered in our own series, though with rather lower means.

It is not possible as yet to ascertain the full significance of the individual variations in our DNAP values ; the various errors inherent in the methods used may account in part for these. Marrow is a very heterogeneous tissue, in which growth is normally achieved by successive mitoses accompanied by differentiation. In some leukæmias, cell proliferation may proceed with practically no differentiation. In any sample of marrow, the number of cells actually in division is never high, although the number of cells preparing for mitosis may be far higher. The effect of a relatively high rate of growth on the average DNAP content per cell cannot, of course, be determined with assurance, but it may be expected to cause an appreciable increase. Indeed, we have found an increase of average DNAP in marrow cells relative to mature blood leucocytes of the order of 19 per cent. in both normal and leukæmic series, this difference being of borderline significance. Variation in chromosome numbers may occur in some cells, though in normal marrow this may be limited to megakaryocytes and occasional endomitotic cells (Schwarz, 1946).

Whatever the impact of these variables, the range of average DNAP values encountered in normal and leukæmic marrow is very similar, and the means show no significant difference. Such similarity in the ranges and means may suggest that there is little fundamental difference in the nuclear organisation of normal and leukæmic cells so far as the deoxyribonucleic acid component is concerned. Mitoses with haploid chromosome numbers have been reported in leukæmic cells by a number of authors (Forkner, 1938 ; Polli, 1950), but the majority of viable cells may well be derived by more normal mitoses. Gunz (1948) found that most mitoses were normal in the early stages of *in vitro* culture of leukæmic blood cells, although some abnormal " resting " cells were seen. Our results would seem to be more consistent with this observation.

The average cellular RNAP is subject to similar wide variation in the normal and leukæmic series, but the mean for the leukæmic series does not differ significantly from the normal mean, though it is a little higher. This is rather surprising, since cytochemical examination of the leukæmic cells shows that the more primitive cells have cytoplasm rich in basophilic material which is removable by ribonuclease. But the cytoplasmic mass of the primitive leukæmic cells is very variable and cases of myeloblastic or lymphoblastic leukæmia often have cells possessing a minimal amount of cytoplasm, although this small amount may be quite basophilic (figs. 4 and 5). In all primitive leukæmic cells, Feulgen-negative nucleoli are prominent and are rich in basophilic material removable by ribonuclease.

A trend linking predominance of primitive cells in the different types of leukæmic marrow with higher average RNAP values per cell is apparent but has not been statistically proved. Similarly, increased cellular maturity following therapeutic remissions is often accompanied by fall in average RNAP and RNAP/DNAP ratios, but the reverse may occur in some acute leukæmias where the primitive cells possess a very small cytoplasmic mass.

The significantly lower mean RNAP for the blood leucocytes as compared with marrow cells reflects the fall in cytoplasmic and nucleolar ribonucleic acid content with maturation, which is apparent on cytochemical examination of the cells. Similarly, although the leukæmic blood cell RNAP is much lower than the leukæmic marrow value, it is still significantly higher than normal blood leucocyte RNAP, and the leukæmic blood samples all contained varying numbers of the more primitive cell types. The general tendency for a fall in RNAP to occur with increased cell maturity is in line with the concepts developed by Thorell (1947) in his quantitative micro-spectrophotometric investigations on hæmopoietic cells.

## SUMMARY AND CONCLUSIONS

1. The average content of deoxyribonucleic acid phosphorus (DNAP) and of ribonucleic acid phosphorus (RNAP) per nucleated cell has been determined in samples of aspirated sternal bone marrow and of venous blood obtained from 22 normal individuals and 17 patients with leukæmia.

2. The DNAP values show a wide range in normal and leukæmic marrow, but the means of the two series are very similar.

3. The mean DNAP is the same in normal and leukæmic peripheral blood leucocytes, but in both series the means are about 19 per cent. lower than those for marrow cells, the differences being of borderline significance.

4. The lack of significant difference between the normal and leukæmic DNAP values is discussed in relation to recent work on the apparent constancy of nuclear DNAP and in relation to mitosis. In so far as the quantity of the deoxyribonucleic acid component relates to nuclear organisation, normal and leukæmic cells do not appear to differ fundamentally.

✓5. The RNAP values show a similar wide range in normal and leukæmic marrow, and the means do not differ significantly.

✓6. The mean RNAP per cell for peripheral blood leucocytes is significantly lower than the corresponding mean for marrow cells both in normal and in the leukæmic series, but the mean RNAP of leukæmic leucocytes is significantly higher than the value for normal leucocytes.

)7. The quantitative studies on the RNAP show a smaller difference between the average amounts in normal and leukæmic marrow cell populations than might be expected from cytochemical investigation.

The great variability of primitive leukæmic cells, particularly in the amount of basophilic cytoplasm present, is discussed in relation to this.

8. In both normal and leukæmic series, the RNAP content of the mature blood leucocytes is lower than that of marrow cells, and a fall in RNAP has also been observed in the marrow cells of some leukæmic patients during the therapeutic remission. This evidence suggests that the ribonucleic-acid content of cells diminishes with increasing maturity, which is in accordance with conclusions drawn from cytochemical examination of the cells.

Our thanks are due to Prof. J. H. Dible and to many colleagues of the Postgraduate Medical School of London for their interest and assistance and for facilities for studying patients. We deeply appreciate the co-operation of the normal volunteers, including members of the Friends' Post-War Service. Drs E. S. Anderson and D. A. Mitchison have kindly advised on the statistical treatment, and Dr C. C. Spicer has scrutinised the results. The photomicrographs were taken by Mr E. V. Willmott, F.R.P.S.

We have to acknowledge with thanks that one of us (I. L.) is the holder of an I.C.I. Research Fellowship in the University of Glasgow and that part of the expenses of this work was met from a grant by the Medical Research Council (to J. N. D.). We wish to thank Mr S. Crowther and Miss M. Meikle for valuable assistance in the analytical procedures.

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5. The RNAP values show a similar wide range in normal and leukæmic marrow, and the means do not differ significantly.

6. The mean RNAP per cell for peripheral blood leucocytes is significantly lower than the corresponding mean for marrow cells both in normal and in the leukæmic series, but the mean RNAP of leukæmic leucocytes is significantly higher than the value for normal leucocytes.

7. The quantitative studies on the RNAP show a smaller difference between the average amounts in normal and leukæmic marrow cell populations than might be expected from cytochemical investigation.

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# DYSTOPIA OF THE NEUROHYPOPHYSIS: TWO CASES

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(PLATES LXXXII-LXXXIV)

ONLY eight cases of the curious condition known as dystopia of the posterior pituitary appear to have been recorded and of these none has appeared in the literature of this country or of America. This degree of rarity and the intrinsic interest of the condition seem to justify the following report of two cases, even though they present no additional features of importance.

## Case 1

### CLINICAL HISTORY

A normally developed parous woman of 57 had been known to have diabetes mellitus for nineteen years. She had been under observation in the diabetic clinic of Hammersmith Hospital under Dr Russell Fraser for eight years, and during this time routine investigations showed nothing to suggest that the diabetes was in any way unusual or that any other endocrine disorder was present. She was stabilised latterly on 26 daily units of protamine zinc insulin. Squamous carcinoma of the right tonsil was diagnosed at the age of 51, was treated with X-rays, recurred four years later, was again treated, and at the time of her death presented a large second recurrence. Death however was not directly due to this, but occurred in hypoglycæmic coma, produced, it was believed, by taking an excessive dose of insulin in the hope of being able to produce sugar-free urine at her monthly visit to the diabetic clinic.

### PATHOLOGICAL FINDINGS

Post-mortem examination two days after death showed nothing unexpected outside the pituitary. There was an ulcerated, infiltrating mass of ill-differentiated squamous carcinoma in the right upper triangle of the neck. The pancreas was grossly normal but had probably some reduction in number of islets. There were early Kimmelstiel-Wilson lesions in the glomeruli of the kidneys; the thyroid and adrenals were normal and the genitalia senile. In removing the brain a firm, pink and slightly bilobed nodule (5×4×4 mm.)

was noted at the apex of the infundibulum. What appeared to be a normal pituitary stalk ran down from it to pierce a normal diaphragma sellæ and join a structure of the size and external shape of a whole normal gland in the pituitary fossa. The nodule with adjoining brain and part of the stalk was embedded whole and sectioned sagittally: the intrasellar gland was sectioned horizontally and both halves embedded. When histological preparations had been examined and the diagnosis made, the remaining material was sectioned serially (at 100 $\mu$ intervals from the upper block and 250 $\mu$ intervals from the lower two). Examination of the intrasellar gland was thus thorough, but that part of the stalk which lay within the diaphragmatic aperture and one lateral half of the upper block were lost.

### Microscopical examination

*The neurohypophysis* forms the nodule noted at the apex of the infundibulum (fig. 1). Histologically it shows little else abnormal. No infiltration with basophils of the type seen near the pars intermedia is present. One single group of epithelial cells (fig. 3) is seen near its upper pole; they are faintly basiphile by Mallory, moderately mucoprotein positive by Pearse's (1949) trichrome-periodic acid-Schiff method, and Sudan-black staining of paraffin sections shows no lipoid.

*The infundibulum* contains no recess but appears otherwise normal. The adjacent structures of the third-ventricle floor, including the supraoptic nucleus, appear normal.

The whole of the apparent stalk of pituitary (fig. 2) is formed by the *pars tuberalis*, which spreads out over the anterior surface of the neurohypophysis and of the infundibulum above it as far as the post-chiasmal angle. At all levels it contains numerous thin-walled blood vessels, running longitudinally; these vessels, which must represent the pituitary portal system, avoid the neurohypophysis almost entirely (fig. 2) though one large channel runs from its posterior surface into the stalk and a few capillary communications can be traced.

The pars anterior appears histologically normal. In view of the post-mortem interval no detailed cytological study was attempted, but Dr A. G. E. Pearse thought the glands normal for a patient of this age. There is no trace of neurohypophysis, pars intermedia or cleft in this part of the gland. The stump of the stalk as it joins the upper surface of the gland consists of pars tuberalis.

## Case 2

### Clinical history

W. S., a male aged 58, was admitted to the London Hospital on 22nd June 1937 with abdominal pain, tenderness and rigidity in the right iliac fossa. At operation, performed immediately, peritonitis was found and a gangrenous appendix removed, with drainage of the peritoneal cavity. He died three days later.

DYSTOPIA OF THE NEUROHYPOPHYSIS

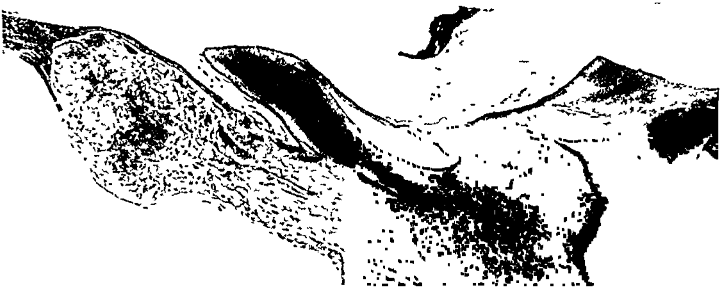

FIG. 1.—Case 1. Mid-sagittal section of region of pituitary stalk, showing the neurohypophysis at the apex of the broad infundibulum, and the narrow stalk formed of pars tuberalis to the left. Hæmalum and eosin. ×5·6.

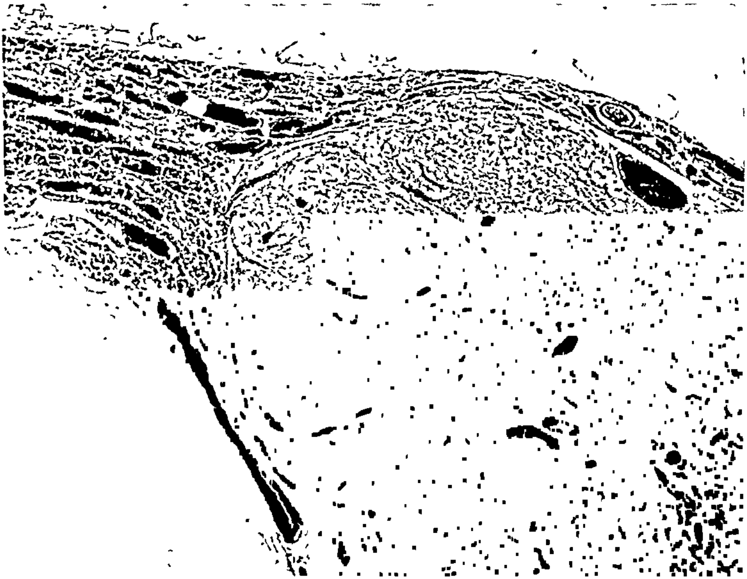

FIG. 2.—Detail of fig. 1, showing the junction of neurohypophysis and pars tuberalis (left), and the prominent portal-system vessels. H. and E. ×33.

TABLE—*Summary of reported cases of dystopia of the neurohypophysis*

| Author | Sex and age | Endocrine disorder | Site of neurohypophysis | Type of tissue uniting two parts of pituitary | Additional pituitary anomalies |
|---|---|---|---|---|---|
| Priesel (1920)<br>Case 1 . . | M. 91 | Pituitary dwarf | Apex of infundibulum | Pars tuberalis | Cystic pars anterior with little functional tissue: partially patent cranio pharyngeal duct |
| Case 2 . . . | M. 67 | None | Immediately above sella | „ | None |
| Priesel (1922)<br>Case 3 . . . | M. 87 | „ | Apex of infundibulum | „ | „ |
| Lhermitte and Roeder (1922) | M. 80 | Diabetes mellitus | „ | Not stated | „ |
| Kraus (1926) . . . | No history given | | Immediately above sella | Pars tuberalis | „ |
| Hamperl (1927-28) . . | M 69 | None | Apex of infundibulum | „ | „ |
| Bosisio (1931) . . . | F. 52 | „ | Attached by a short pedicle to the left anterior aspect of the pituitary stalk | Normal pituitary stalk (?) | Nodules of what are described as pars intermedia (possibly tuberalis) lay in contact with both neuro- and adenohypophysis |
| Biemond and Harz (1935) . | F. 31 | Genital hypoplasia (ovarian dwarf) | Floor of third ventricle | None | Pars tuberalis lay in contact with the neurohypophysis, but was entirely detached from the pars distalis |
| Present series<br>Case 1 . . | F. 57 | Diabetes mellitus | Apex of infundibulum | Pars tuberalis | None |
| Case 2 . . . | M 58 | None | „ | „ | „ |

## PATHOLOGICAL FINDINGS

Post-mortem examination (P.M. 265, 1937) performed 16 hours after death revealed general sero-fibrinous peritonitis as the cause of death. The subject was a tall (6 ft.) well-developed stout muscular man. Incidental findings were the replacement of the right suprarenal by confluent fibrocaseous tuberculous nodules, focal hyperplasia and atrophy of the cortex of the left suprarenal, and obsolete tuberculosis of the sub-apical region of the right lung and of the right hip-joint. Two colloidal adenomata were present in the otherwise normal thyroid gland. The first part of the duodenum showed two shallow peptic ulcers.

The stalk of the pituitary from the infundibulum to the level of the diaphragma sellæ was expanded by a solid grey nodule (0·7 cm. in diameter). A short segment of stalk united its lower extremity to the anterior lobe (weight 0·4 g.) in the sella turcica. The anterior lobe was of normal appearance; no trace of posterior lobe tissue was visible in its border.

## MICROSCOPIC EXAMINATION

On mid-sagittal section the nodule in the stalk (fig. 4) is composed of tissue identical in appearance with that of the normal posterior lobe of the pituitary. No epithelial cells are present, as in case 1. It is coated anteriorly with a narrow strip of pars lingualis. A small group of cells belonging to the pars tuberalis lies in the posterior angle between the nodule and its attachment to the infundibulum.

After fixation in formaldehyde the anterior pituitary was divided sagittally into four portions which were embedded together in paraffin wax. Microscopically there is no histological abnormality, and no trace of posterior lobe is present. The lower extremity of the stalk is represented by a tag of tissue resembling the normal pars lingualis.

## DISCUSSION

Reported cases of this anomaly, including the present two, are collected in the table. Congenital anomalies of the pituitary are rare, only the various forms of craniopharyngeal duct persistence and the lesions associated with anencephaly being even relatively frequent. The only reported cases found which bear even remote relation to the condition under discussion are Kraus's second case (1926), a child of 14 days with neurohypophysis embedded in the anterior surface of the pars anterior—no clinical details and no details of stalk anatomy are given, and Ratzenhofer's case (1938) in which a deformity of the basis cranii displaced the whole pituitary into the cranial cavity.

The most remarkable feature of the tabulated cases is the lack of any characteristic endocrine effect. Lhermitte and Roeder's case (1922) and our first case both had diabetes mellitus; an incidence

DYSTOPIA OF THE NEUROHYPOPHYSIS

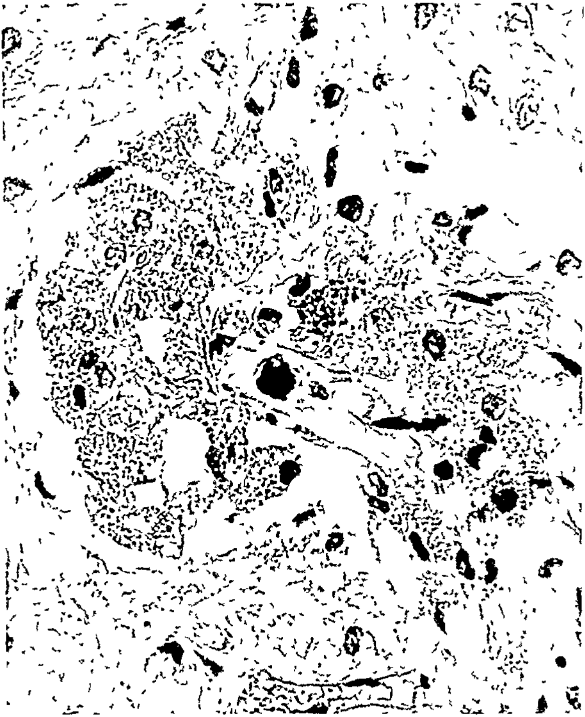

FIG 3—Case 1 The one group of epithelial cells seen in the neurohypophysis Periodic acid Schiff orange G ×580

of two out of ten of a condition whose general incidence at the relevant ages is not over 1 per cent. could be shown to be statistically significant, but it seems wise to withhold judgment on this in so small a series. Two cases did, of course, have major endocrine disorders but in both the pituitary lesion was more than a simple dystopia. In Priesel's first case (1920) the functional pars anterior was greatly reduced by the cyst, and it cannot be doubted that this was the effective lesion. In Biemond and Harz's case (1935) the moderate dwarfism was believed to be ovarian in origin, and the ovarian hypoplasia was ascribed by the authors to the pituitary lesion. If this is so—and the case is not an easy one to assess—a comparison with other cases suggests that the interruption of the pituitary stalk, and with it the portal system, was more important than the displacement of the neurohypophysis, though this was more extreme than in any other case. In general, it seems safe to assume that simple dystopia of the type seen in the two cases described in the present article does not lead to any change in pituitary function.

The constancy of the juxtaposition of the neuro- and oro-hypophysis in vertebrates has encouraged the belief that this is of functional significance. Support may be advanced for such an argument through the frequency of "invasion" of the neurohypophysis by trabecular masses of basophil cells and, more doubtfully, through the presence of "colloid" vesicles amongst the borderline cells. Neither of these features has, however, been proved to be of functional significance, and it has never been demonstrated with certainty that the contiguity of the two parts is necessary to any of the known physiological functions of the pituitary. In whales (Geiling and Robbins, 1938) a fibrous septum between the lobes affects the function of neither of them. In the wood-frog, Atwell (1938) succeeded in displacing the embryonic pars anterior from the floor of the third ventricle without disturbing its differentiation or any detectable part of its function. These cases of dystopia of the neurohypophysis afford additional evidence that normal contact between neuro- and oro-hypophysis and, incidentally, differentiation of the pars intermedia, is not necessary for the function of the pituitary in man. It is true that the pars tuberalis remained in contact with the pars nervosa in all cases, but there was no histological evidence of activity along the line of contact, and if the pars tuberalis has any significance in these cases it may well be chiefly in virtue of the portal vessels which it carries. It may be concluded that the association of the two halves of the pituitary is simply an accident of developmental contiguity perpetuated by the hypothalamic connections of the pars anterior.

## SUMMARY

In the two cases described the pars nervosa of the pituitary was found accidentally at post-mortem to lie between the infundibulum

and the diaphragma sellæ, being connected to the normal pars anterior lying in the pituitary fossa by a stalk consisting of pars tuberalis. One patient had diabetes mellitus, the other no known endocrine disorder. Ten cases of this condition have now been described. When uncomplicated it does not appear to result in any disorder of pituitary function.

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DYSTOPIA OF THE NEUROHYPOPHYSIS

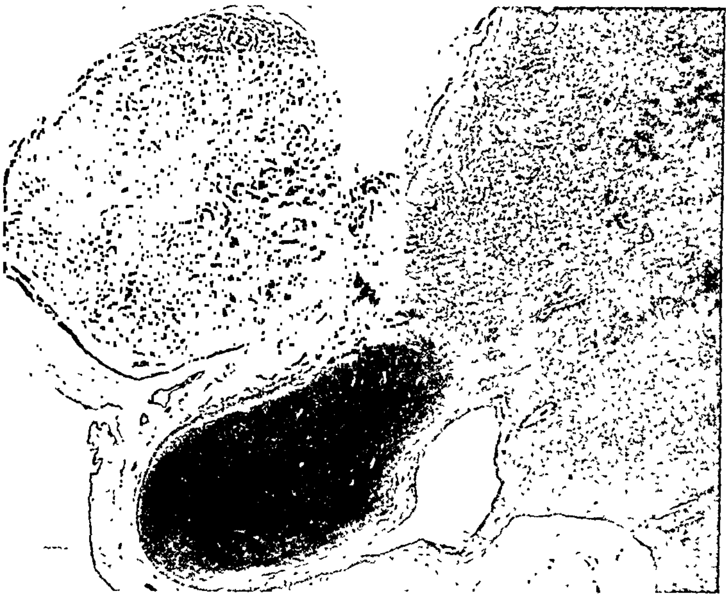

FIG. 4.—Case 2. Mid-sagittal section showing the optic chiasma and nodule. H. and E. ×9.

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# CHOLESTEROL CONTENT OF TABETIC SPINAL CORD

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(Plate LXXXV)

The lipids of myelin consist largely of free cholesterol, sphingomyelin and cerebroside, in Wallerian degeneration this free cholesterol disappears and there is also a fall in total cholesterol (Johnson, McNabb and Rossiter, 1948, 1949). A similar fall in cholesterol is likely to accompany the pronounced loss of myelin in tabes dorsalis where demyelination is histologically confined to the posterior white columns of the spinal cord as well as the dorsal roots. It appeared of interest to establish by simple chemical technique if there is, in fact, a fall in cholesterol content of the posterior columns. Accordingly, we estimated the cholesterol content of both posterior and lateral columns from four normal spinal cords and also from one cord showing severe and extensive tabes dorsalis.

## Material and methods

*Material.* Four normal spinal cords (cords A, B, C and D—table I) and one cord from a case of tabes dorsalis (cord E—table II) were used. Cord A was from a man aged 66 who died from congestive heart failure following chronic rheumatic heart disease, cord B was from a man aged 48 who died from cardiac infarction, cord C from a man aged 25 who died from cerebral abscess following chronic otitis media and cord D from a man aged 55 who died from carcinoma of the ascending colon Cord E was from a man aged 69 who had suffered from tabes dorsalis. He was said to have contracted syphilis at the age of 20 Severe tabes was diagnosed at age of 65, and aortic incompetence was found at the age of 67. The Wassermann reaction of the C.S F. was strongly positive shortly before death from generalised peritonitis following painless acute appendicitis. Post mortem examination showed extensive tabes dorsalis and syphilitic aortitis. The cords were fixed in 10 per cent. formol saline and pieces were taken for analysis after varying periods up to 13 months (see tables I and II). According to Brante (1949) the cholesterol content of white matter does not change during prolonged immersion in formaldehyde solution. Our results confirm this view for the white matter of spinal cord (cords A and E), examined at intervals after a period of 3 to 13 months of storage, but in two other cords (C and D), a slight fall in cholesterol appeared to occur during the first three months of storage. Various possible factors may account for this slight diminution during the early stage of storage However, the conclusions, based largely on the value of the ratio of cholesterol content of the posterior and lateral columns, could not be affected by so slight an alteration. Furthermore, the mean absolute values cited in the text for normal cord A, and for tabetic cord E, refer to observations made after three months or more of storage.

*Preparation of tissue.* For each estimation a cylindrical piece of lumbar or thoracic cord 1-2 cm. long was removed by transverse cuts with a mounted razor blade. From this piece of cord portions of both posterior and lateral tracts were obtained. Firstly, the pia-arachnoid was stripped from the surface of the cord. The moistened, transversely cut surface of the cord was then examined with a hand lens so as to orientate the razor before incision and a longitudinal incision was made starting just medially to the points of entrance of the dorsal roots. The incision was directed anteromedially to meet a similar incision from the opposite side. In this way, a piece of white matter (sectorial in transverse section) was cut from the posterior columns. Similar strips of white matter were then cut from the anterolateral tracts of both sides. Great care was taken during these procedures to ensure that no grey matter was included in the strips used for analysis. The cord remaining after removal of the strips of white matter consisted of a piece of tissue, stellate in transverse section, containing all the grey matter and remnants of the white. In many cases the stellate pieces were later examined histologically to check the accuracy of excision (see fig. 2).

*Extraction of tissue.* The pieces of posterior and anterolateral columns excised in the above manner were rinsed with a jet of distilled water on filter paper, then blotted and dropped into 100 ml. pyrex flasks containing 20 ml. ethanol-ether mixture (3 vols. ethanol : 1 vol. diethyl ether). Each flask was stoppered with a ground glass stopper and left in the refrigerator overnight. Next morning the pyrex flasks were connected by ground glass joints to reflux condensers and heated on a hot plate with reflux condensation for 2 hrs. The supernatant fluid was then poured off from the extracted pieces of tissue into "Quickfit" flasks. The original flasks containing the pieces of tissue were washed with 3 × 5 ml. washings of ethanol-ether, the washings being transferred to the "Quickfit" flasks. The pieces of tissue in the pyrex flasks were then re-extracted with a further 20 ml. ethanol-ether for 2 hrs. The pieces of tissue were removed from the second extract and transferred to pyrex micro weighing glasses, dried for 3 hrs. at 100° C. and weighed on an air-damped semi-micro balance. The fat-free dry weights were generally between 4 and 11 mg. The "Quickfit" flasks containing the first extracts of cord, together with the washings, were evaporated *in vacuo* to small volume on an electric hot plate. The final fluid was removed by raising the flask from the hot plate and sucking air through the hot flask to blow off the remaining solvent, great care being exercised to avoid overheating while the final fluid was being removed. The flasks were then placed in a desiccator until ready for estimation. In several experiments the fluid from the second extraction was similarly treated, but in no case was any significant amount of cholesterol detected. It was therefore concluded that the first extraction removed all the cholesterol, but the second extraction of the tissue was always carried out so as to obtain strictly comparable values of fat-free dry weight.

*Estimation of cholesterol.* The dry residues in the "Quickfit" flasks were dissolved in 25 ml. of boiling chloroform and transferred quantitatively with 2 × 10 ml. washings into 50 ml. glass-stoppered volumetric flasks. After cooling, the solutions in the flasks were made up to 50 ml. with chloroform. Five ml. of this solution were transferred to a 25 ml. glass-stoppered graduated cylinder and the volume was made up to 10 ml. with chloroform, except in the case where 10 ml. were used for the posterior columns of the tabetic cord.

Standards containing 0·25 mg. and 0·5 mg. cholesterol in 10 ml. chloroform, prepared daily from a stock solution of cholesterol in chloroform of strength 100 mg./100 ml., were placed in other 25 ml. glass-stoppered cylinders. Two ml.

sulphuric acid were added to 40 ml. acetic anhydride, the mixture being cooled in a refrigerator for about 10 minutes. Four ml. of this reagent were added in turn at 3 minute intervals to each of the solutions of cholesterol in chloroform, first to the standards and then to the unknowns. The cylinders were shaken and placed in a water bath at 20° C. in the dark. After exactly 30 mins each cylinder was removed and the intensity of the green colour which had developed was measured in a photoelectric colorimeter using a red filter. The "blank" consisted of a similar mixture of chloroform (20 ml.) and acetic anhydride-sulphuric acid mixture (8 ml). The blank mixture was first placed in two optically matched test tubes of an Evans electroselenium photoelectric colorimeter or in two matched fused cells of a similar Unicam instrument. Readings were taken to ensure equality in optical density of the two blanks One of the blanks was then discarded and its tube or cell was filled successively with the various green cholesterol solutions for estimation. The other blank was read before and after each reading to ensure constancy in galvanometer deflection. Each reading was made in duplicate for each solution. The changes in deflection measured on a logarithmic scale were found to be directly proportional to the amount of cholesterol present. Known standards were included in every batch of estimations.

The above is a modification of the method of Sperry and Brand (1943). Saponification of cholesterol esters was not carried out This might have diminished the results by as much as 10 per cent if all the cholesterol were present in ester form, since cholesterol esters give a slightly more intense colour with acetic anhydride and sulphuric acid than free cholesterol (Sperry and Brand), however, Rossiter and his colleagues have shown that nearly all the cholesterol of normal white matter is free cholesterol so that little was to be gained by saponification which would have involved further manipulations with possible loss It would, of course, be of interest to know what proportion of the cholesterol of tabetic posterior column is free cholesterol, but even if all the remaining cholesterol is present as esters this would only have the effect of making the very low recorded values a little lower still

*Recovery of cholesterol.* The amounts of cholesterol extracted varied from 1-5 mg. depending on the amount of tissue excised. Solutions containing respectively 2 mg. and 5 mg. of cholesterol dissolved in 20 ml ethanol ether were refluxed for 2 hrs. and then evaporated to dryness and dissolved in chloroform as in the treatment of the extracts of spinal cord. Estimation showed these solutions to contain 1 89 and 4 7 mg. of cholesterol, representing a recovery of 94·5 and 94 per cent respectively. In another experiment using 2 mg. of cholesterol the recovery was 97 per cent.

*Histological technique.* Blocks of each spinal cord were mordanted in bichromate fluoride for 5 7 days and then embedded in paraffin-wax. Sections were cut and stained for myelin by the Weigert-Pal method and by Sudan black. Sections were stained for 7 mins. with a saturated solution of Sudan black in 70 per cent. ethanol; after washing off the stain with 70 per cent. ethanol the sections were mounted in glycerol-jelly. This convenient adaptation of Ciaccio's method (Lison, 1936) gives a very similar picture to that obtained by the more lengthy Weigert-Pal procedure; furthermore, it more rationally demonstrates the lipids of the myelin sheaths Fig. 1 shows a section of normal spinal cord stained by Sudan black after Weigert mordanting.

When pieces of spinal cord were examined after excision of the tracts, routine staining with Ehrlich's hæmatoxylin and eosin was employed to ensure that the grey matter was intact In two experiments with normal spinal cords the pieces remaining after excision of the tracts were examined by the Sudan black procedure. Fig. 2 shows a section fromt his material which illustrates the method of excision.

## RESULTS

Considerable diminution in stainable lipid accompanied the loss of myelin in tabes dorsalis. Fig. 3 is a photograph of a section of the tabetic cord stained by Sudan black, and fig. 4 shows the same tissue stained by the Weigert-Pal method. It is apparent that the demyelination of the posterior columns brings about a most pronounced loss of lipid. On the other hand, sections from all the four normal cords showed uniform intensity of coloration of both posterior and lateral columns by both methods (fig. 1).

In tabes dorsalis a marked fall in cholesterol content accompanied the loss of myelin. Table I gives the results of analysis of the four normal spinal cords A, B, C and D. The mean amounts of cholesterol in the posterior columns were respectively 0·46, 0·52, 0·59 and 0·53 mg./mg. fat-free dry weight. In the tabetic cord (see table II) the cholesterol content was constantly much lower, averaging 0·20

TABLE I

*Cholesterol content of four normal spinal cords*

| *Spinal cord* | Experiment no. | No. of months *in* formol-saline | Level of piece *of cord* analysed | *P* | *L* | *P/L* |
|---|---|---|---|---|---|---|
| A | 1 | 3 | lumbar | 0·45 | 0·38 | 1·18 |
| | 4 | 6 | thoracic | 0·45 | 0·43 | 1·05 |
| | 6 | 7 | ,, | 0·47 | 0·46 | 1·02 |
| | 8 | 7 | ,, | 0·45 | 0·53 | 0·85 |
| | 10 | 7 | ,, | 0·45 | 0·54 | 0·83 |
| | 14 | 11 | ,, | 0·49 | 0·54 | 0·91 |
| | Mean | | | 0·461 | 0·479 | 0·97 |
| B | 11 | 0·25 | thoracic | 0·54 | 0·44 | 1·23 |
| | 12 | 0·25 | lumbar | 0·51 | 0·39 | 1·30 |
| | Mean | | | 0·525 | 0·415 | 1·26 |
| C | 15 | 0·3 | thoracic | 0·61 | 0·64 | 0·95 |
| | 16 | 0·3 | ,, | 0·62 | 0·61 | 1·02 |
| | 21 | 3 | ,, | 0·56 | 0·52 | 1·08 |
| | 22 | 3 | lumbar | 0·59 | 0·46 | 1·28 |
| | Mean | | | 0·595 | 0·557 | 1·08 |
| D | 19 | 0·5 | thoracic | 0·56 | 0·66 | 0·85 |
| | 20 | 0·5 | ,, | 0·62 | 0·63 | 0·98 |
| | 23 | 3 | ,, | 0·44 | 0·52 | 0·87 |
| | 24 | 3 | ,, | 0·52 | 0·45 | 1·16 |
| | Mean | | | 0·535 | 0·565 | 0·96 |

Each experiment refers to analysis of posterior and lateral columns excised from a piece of cord 1-2 cm. long.

P represents mg. cholesterol/mg. fat-free dry weight of posterior column.

L represents mg. cholesterol/mg. fat-free dry weight of lateral column.

CHOLESTEROL CONTENT OF TABETIC SPINAL CORD

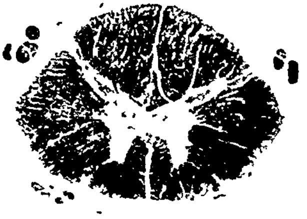

FIG 1—Normal spinal cord from case C Sudan black ×6

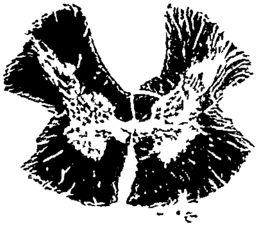

FIG 2—Normal spinal cord from case C after excision of tracts for analysis Sudan black ×6

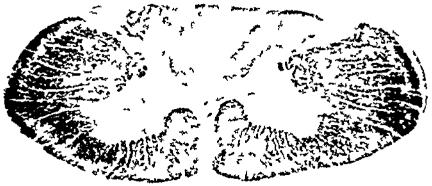

FIG 3—Tabetic spinal cord from case E Sudan black ×6

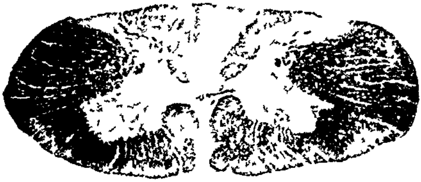

FIG 4—Tabetic spinal cord from case E This section was from the same block as fig 3 Weigert Pal ×6.

mg./mg. fat-free dry weight. The cholesterol contents of the lateral columns averaged 0·48, 0·41, 0·56 and 0·56 mg./mg. fat-free dry weight respectively for the same four normal spinal cords, and 0·37 mg./mg. fat-free dry weight for the tabetic cord.

Unfortunately, only one tabetic cord was available. However, the highly consistent diminution of cholesterol content of all the samples of the posterior columns of the tabetic cord, to less than one half of the values found for the posterior columns of the normal cords (tables I and II), justifies the conclusion that the change is a significant chemical concomitant of the loss of myelin. The figures suggest that there may be also a slight fall in cholesterol content of the lateral columns in tabes but the figures are more variable and the difference is much less pronounced than in the posterior columns.

TABLE II

*Cholesterol content of tabetic spinal cord*

| Spinal Cord | Experiment no | No of months in formol saline | Level of piece of cord analysed | P | L | P/L |
|---|---|---|---|---|---|---|
| E | 2 | 3 | lumbar | 0·20 | 0·36 | 0·56 |
| | 3 | 5 | thoracic | 0·21 | 0·36 | 0·58 |
| | 5 | 7 | ,, | 0·20 | 0·35 | 0·57 |
| | 7 | 7 | ,, | 0·22 | 0·40 | 0·55 |
| | 9 | 8 | ,, | 0·22 | 0·37 | 0·59 |
| | 13 | 11 | ,, | 0·22 | 0·39 | 0·56 |
| | 17 | 13 | ,, | 0·21 | 0·40 | 0·53 |
| | 18 | 13 | cervical | 0·15 | 0·35 | 0·43 |
| | Mean | | | 0·204 | 0·372 | 0·55 |

Each experiment refers to analysis of posterior and lateral columns excised from a piece of cord 1·2 cm long

P represents mg cholesterol/mg fat free dry weight of posterior column

L represents mg cholesterol/mg fat free dry weight of lateral column.

The ratio of the cholesterol content of the posterior column to the cholesterol content of the lateral column (P/L ratio) bears out this conclusion (tables I and II). Dual samples from posterior and lateral columns were always analysed simultaneously and thus the use of the ratio eliminates any errors due to slight day-to-day variation in technique. The P/L ratio for normal cords (16 dual samples from 4 cords) varied from 0·83 to 1·30, the over-all mean being 1·035. In tabetic cord (8 dual samples from 1 cord) the value of P/L was 0·43-0·59 with an average of 0·55. Mr N. T. J. Bailey has kindly examined the P/L values by statistical methods and finds that among the four normal cords A, B, C, D the variation *between* cords is no greater than the variation *within* cords. Thus the 16 values of P/L can be regarded as a homogeneous group. The one tabetic cord E shows much less variation (in fact a little more than 1/10 of the normal) among its 8 values of P/L. In order to attempt a comparison between the single tabetic cord and the normal cords we might assume that the variation between different severely tabetic cords is no greater than the variation in normal cords. Thus comparing the tabetic mean (0·55), *regarded as a single observation*, with the mean of the 16 normal cords (1·035), the tabetic average is significantly lower than the normal average ($P < 1$ per cent.).

It might be held that diminution in the cholesterol content of the posterior columns in tabes, expressed on the basis of fat-free dry weight, results from increase in the fat-free dry weight owing to replacement of myelin by glial tissue, without any absolute decrease in cholesterol. It is, however, extremely improbable that the fat-free dry weight of the posterior columns could be increased in tabes when there is at the same time such pronounced wasting of these tracts. To test this point further, we have measured the cholesterol content and the fat-free dry weight of the entire posterior columns of pieces of normal and tabetic cord, each 1·0 cm. in length. The whole posterior columns were excised from two cylindrical pieces of mid-thoracic cord, each 1·0 cm. in length, taken from cord A (normal) and cord E (tabetic) ; the complete tracts were readily gouged out with a U-shaped incision aided by spontaneous fission along the posterior horns and the commissure of the grey matter. At this level of the spinal cord the grey matter is extremely scanty, so that the quantity of grey matter included in the excised pieces would be negligible. The fat-free dry weight of the entire posterior columns from each 1·0 cm. length of cord was 15·5 mg. for cord A (normal) and 8·3 mg. for cord E (tabetic). The cholesterol content expressed in mg./mg. fat-free dry weight was 0·46 for the normal posterior columns and 0·17 for the tabetic posterior columns, while expressed in mg./cm. of whole posterior columns, the cholesterol values were respectively 6·8 for the normal cord and 1·45 for the tabetic cord. Thus the fraction of the cholesterol lost in tabes for each 1·0 cm. length of entire posterior columns is considerably greater than the fraction lost per mg. fat-free dry weight, owing to the fall in fat-free dry weight of the whole tabetic posterior columns.

It is therefore clear that the low values for cholesterol content of the posterior columns observed in this severe case of tabes dorsalis, when expressed in mg./mg. fat-free dry weight, give an extremely conservative estimate of the loss of cholesterol. That these values are indeed so low in comparison with those for normal posterior columns and also as compared with the values for tabetic lateral columns, emphasises the magnitude of the loss of cholesterol from the posterior columns in tabes. This profound loss of cholesterol thus reflects the outstanding histological and histochemical features of the disease,

## SUMMARY

1. Samples of the posterior columns of a tabetic spinal cord contained consistently less than one half the cholesterol found in similar samples from four normal spinal cords.

2. The amounts of cholesterol in both posterior and lateral columns in each of 16 cylindrical pieces of normal spinal cord were determined. The mean ratios of the cholesterol contents of posterior to lateral columns averaged 1·035. The same ratio averaged 0·55 for 8 similar cylindrical pieces of a tabetic cord.

We are grateful to Professor H. R. Dean for his interest in this work and to Dr A. M. Barrett and his staff for their helpful co-operation in providing us with material. Mr N. T. J. Bailey, Statistician to the Department of Medicine, has given us valuable advice on the results, and provided the statistical analysis. We also wish to thank Mr D. Madin for his competent technical assistance and Mr S. Patman for the photomicrography.

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616—006 . 34 . 04 : 599 . 812 . 2 (*Lemur catta*)

# A SARCOMA OF BONE IN A RING-TAILED LEMUR (*LEMUR CATTA*)

R. Warwick

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(Plates LXXXVI-LXXXVIII)

The animal which is the subject of this report, a female lemur (*Lemur catta*), was on exhibition in the Zoological Gardens, Belle Vue, Manchester. The advice and help of this department was sought on account of a large and chronic swelling of the animal's left wrist. It was soon evident that the mass was a neoplasm of some kind, and it proved to be an osteosarcoma.

Fox (1923), in a series of 5365 autopsies on mammals and birds in the Philadelphia Zoological Gardens, encountered only 94 neoplasms, 1 of which, a papillary adenoma of the prostate, occurred in a lemur. In an earlier series, Fox (1912-13) had seen no tumours at autopsies on 328 primates and 60 lemurs. Ratcliffe (1933) extended the Philadelphia records up to 1932, incorporating the earlier reports of Fox (1923). Including lemurs, 1555 primates in all were examined after death and 11 tumours were observed. Only one of those reported by Ratcliffe (1930) was derived from bone, a giant-cell tumour in a chacma baboon (*Papio porcarius*). Ratcliffe draws attention to the rarity of bone tumours in captive wild animals as a whole. White and Fox (1910) recorded an osteochondroma in a cat. The Annual Reports of the Pathologist of the London Zoological Society indicate a very low incidence of neoplastic disease. Plimmer (1910) reported a renal sarcoma in a lemur, and Scott (1926) discovered multiple hydatid cysts in the lungs of another specimen (*Lemur catta*), but nothing resembling the neoplasm recorded here appears to have been recorded in a lemur, nor, indeed in any primate.

## Case report

The animal reached Belle Vue Gardens in September 1946, when its age was estimated at approximately 18 months. It remained active and in good health until June 1949, when a small double swelling was noted on the dorsum of the left carpus. This was at first considered to be an abscess. It remained stationary in size for some months and appeared to interfere little with the animal's activities.

During April 1950, about 10 months after its appearance, the tumour was noted to be enlarging rapidly. A further period of growth was noted in July 1950, and by this time it had become large and heavy enough to impede movements. The lemur was also seen to be feeding poorly, suggesting some effect upon her general health. By September 1950, the tumour had become so enormous and the animal so miserable that it was transferred to this department for examination and, if practicable, operation.

When first seen the lemur was docile, inactive, sluggish in movement, and had completely lost the lively and acrobatic behaviour typical of its kind. It moved only when necessary, obviously finding the mass on its left forearm a great embarrassment, which was hardly surprising in view of the tumour's weight (250 g.) compared with that of the animal (2·22 kg.). The left manus was kept tightly clenched and was not used for grasping. In walking the creature's weight was rested upon the dorsum of the tumour, a practice which had created an area of excoriation. Appetite was capricious, and the lemur whimpered frequently, as if in almost continuous pain or discomfort.

The appearance of the tumour is shown in fig. 1. The mass surrounded the lower forearm, carpus, and partially the metacarpus, the remaining part of the hand appearing as an excrescence upon the distal aspect of the tumour. On palpation the mass was very firm, slightly elastic, and contained in its dorsal part an irregular area of bony hardness. The skin was tense and excoriated from a circular area on the dorsum of the tumour. The whole mass was slightly mobile. It appeared to be limited to the lower forearm and carpus. No secondary tumours were detected in the axilla or elsewhere by palpation.

In X-ray photographs the density of the neoplasm obscured the bones involved within it, but comparison with the normal limb suggested changes in the radius, ulna and carpus (fig. 2). There was no radiological evidence of metastasis.

A provisional diagnosis of osteochrondroma or osteosarcoma was made. In view of the absence of metastases, and to satisfy all those interested that no trial had been rejected to save the limb, it was decided to explore the tumour. This was done by Mr A. F. Williams, under light intravenous nembutal anæsthesia. The mass ensheathed the bones and soft tissues of the lower half of the forearm, the entire carpus, and proximal metacarpus. It was continuous with these bones, and the forearm flexors and extensors disappeared into its depths. The bulk of the tumour was clipped away leaving the bones and muscles intact as far as possible. This procedure left portions of the tumour tissue in the distal part of the interosseous space and carpus, the constituent elements of which were partially obscured. Owing to this disorganisation of the carpus the hand could be moved *en masse* from side to side relative to the forearm. It was obvious

## OSTEOSARCOMA IN A LEMUR

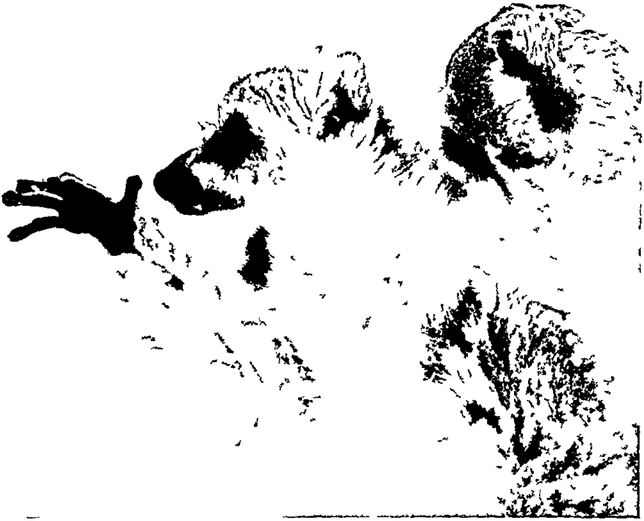

FIG 1 —Anterior aspect of the tumour which engulfs the lower forearm and carpus the manus projecting beyond the mass The skin of the palm is stretched and shining

OSTEOSARCOMA IN A LEMUR

FIG 2—Radiograph of the neoplasm *in situ* showing even density except over the dorsum of the carpus where a denser area of bone formation is visible The hand is superimposed upon the tumour

FIG 3—Radiograph after resection of the bulk of the tumour showing disorganisation of the carpus and invasion of the lower end of the radius

OSTEOSARCOMA IN A LEMUR

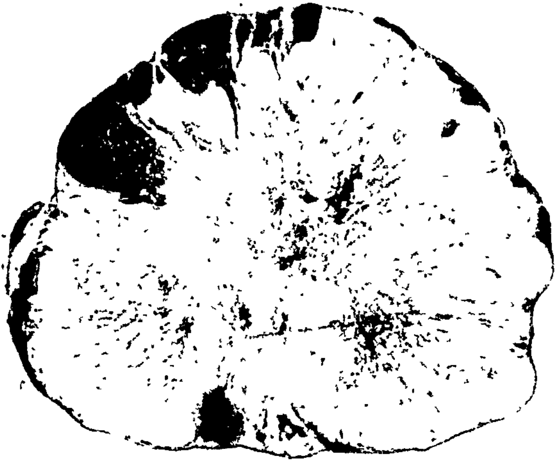

FIG. 4.—Cut surface after bisection of the neoplasm showing superficial blood cysts at periphery, a central area of bone and the main mass of non-calcified osteoid tissue. c. natural size.

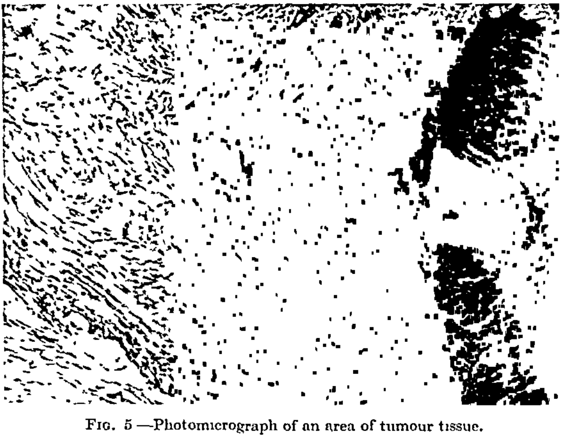

FIG. 5.—Photomicrograph of an area of tumour tissue. Hæmalum and eosin. ×125.

that further resection, if sufficiently radical, would mutilate the extremity beyond hope of any functional result, and therefore a lethal dose of nembutal was administered before recovery from the anæsthetic

Autopsy confirmed the involvement of the bones of the left fore-extremity A radiograph showed disorganisaton of the carpus and invasion of the lower end of the radius (fig 3) No secondary tumours were found and the lymph nodes of the left axilla were not involved X-ray photographs of the extirpated tumour showed an area of diffuse calcification near its centre, but none at the periphery On section the main mass of the tumour consisted of non-calcified osteoid tissue with a central area of bone (fig 4).

The following report upon the histology of the tumour was supplied by Professor S L Baker

*Osteosarcoma* Sections show a tumour composed mainly of non-calcified osteoid tissue arranged as a spongework of closely-packed irregular trabeculæ The spaces between the trabeculæ contain vessels, osteoblastic cells and a fair number of multinucleated osteoclasts The osteoblastic tissue shows cytological evidence of malignancy and the osteoid tissue shows a much greater cell content than normal The general appearances are similar to those seen in some human bone sarcomas (fig 5)

## SUMMARY

An osteosarcoma involving the radius and carpus was found in a lemur (*L catta*) from the Belle Vue Zoological Gardens at Manchester No secondaries were detected clinically or radiologically An attempt was made to remove the neoplasm, but it was seen that amputation would have been necessary, and the lemur was destroyed by an overdose of anæsthetic Autopsy and microscopic examination of the tumour confirmed the diagnosis of osteosarcoma.

The author is indebted to Professor G A G Mitchell for his interest and help, to Professor John Morley for his surgical advice, to Mr A F Williams for attempting removal of the neoplasm, and to Professor S L Baker for his pathological report Mr Gerald Iles, Superintendent of the Belle Vue Zoological Gardens, kindly permitted him to make this report and furnished the details of the animal s history Thanks are due to Mr P Howarth and Mr F L Newell for the photographs and radiographs

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# MYXŒDEMA: AN AUTOPSY REPORT WITH HISTOCHEMICAL OBSERVATIONS ON THE NATURE OF THE MUCOID INFILTRATIONS

Douglas B. Brewer

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(Plates LXXXIX and XC)

Since the Report of the Committee of the Clinical Society of London on myxœdema published in 1888 there have been few reports of autopsies on untreated cases of myxœdema probably because treatment of the disease with thyroid extract was introduced by G. R. Murray of Manchester in 1891.

Means (1948) reviewing the experience of the Thyroid Clinic of the Massachusetts General Hospital could report records of only 9 post-mortem examinations on cases of myxœdema in 30 years and only 3 of these were on untreated cases. During this time 600 cases of myxœdema had passed through the clinic. Most of the published reports are of single cases, many of them treated. The outstanding exception is the paper by Bastenie (1944) giving an account of 10 untreated cases.

As a consequence of this paucity of anatomical material there are still many unanswered problems regarding the disease. The present study was directed particularly to the nature of the so-called mucoid infiltrations and the changes in the myocardium.

## Clinical history

The patient was a man of 56 who, for 10 years, had suffered from weakness, lethargy, breathlessness and constipation. Clinically he presented the picture of an obvious, advanced case of myxœdema.

The basal metabolic rate was minus 34 per cent. The serum cholesterol was 400 mg. per 100 ml., the total serum proteins 7·25 g. per 100 ml. and serum albumin 5·2 g. per 100 ml. The electrocardiogram showed sinus rhythm with a rate of 63 per min., ventricular extra-systoles and left ventricular preponderance.

The patient was given thyroideum (B.P.) gr. ¼ t.d.s. for one day. Early the next morning he suddenly collapsed and died in a manner suggesting *coronary thrombosis*.

## Post-mortem findings

The body was that of a pale obese man with a protuberant abdomen. Both hands and feet were slightly swollen. There was only slight loss of hair on the eyebrows. Both eyes showed a mild purulent conjunctivitis. There was œdema of the subcutaneous and retroperitoneal tissues and slight subperitoneal œdema showing especially over the stomach. The œdema fluid was clear and was not easily expressed from the tissues. There were moderate bilateral pleural effusions and a pericardial effusion, all of clear yellow fluid.

The *heart* was enlarged (wt. 600 g.) mainly owing to marked left ventricular hypertrophy. The tricuspid, pulmonary and mitral valves were normal but the aortic valve showed marked calcification of all its cusps. The *coronary arteries* showed patchy atheroma; there was no occlusion. The *aorta* showed moderately severe, but rather patchy, atheroma throughout its length and in its main branches.

The *thyroid* was small (wt. 20 g.) with thin fibrotic lateral lobes in which were scattered a few small nodules of surviving thyroid parenchyma. The *adrenals* (combined wt. 15 g.) and *pituitary* were macroscopically normal. The thymus was almost completely atrophied to fatty tissue.

The *tongue* was large but its epithelial covering appeared normal. The *œsophagus* was normal. The *stomach* had a number of raised circular patches in the mucosa, 0·5-1 cm. in diameter, most numerous along the lesser curve. The *small intestine* was normal. The *pelvic colon* appeared longer than normal and was folded sharply on itself several times. The *liver* (wt. 2500 g.), *gall bladder* and *pancreas* were normal and the *spleen* (150 g.) showed slight lymphoid hyperplasia.

The *epiglottis* and *larynx* were slightly œdematous. The *trachea* and *main bronchi* were normal. There was atrophic emphysema of the *lungs* with congested lower lobes and a small calcified scar at the apex of the left lung.

The *genito-urinary system* was normal except for small bilateral hydroceles.

The *cerebral vessels* showed severe atheroma in the form of numerous closely set discrete nodules especially along the basilar artery. The brain and meninges were normal.

## HISTOLOGY

There was very slight patchy increase in fibrous tissue in the *cardiac muscle* but the fibres generally were not more separated than normal. In a few fibres the central area was replaced by a slightly granular amorphous mass of faintly basophilic material which replaced the sarcoplasm in the centre of the fibre and extended along its length being bounded on either side by a thin band of normal sarcoplasm (fig. 1). The deposits occurred in varying relationship to the nucleus and occasionally extended along a branching connection from one fibre to the next.

The epithelium of the *tongue* was slightly thickened and the subepithelial tissues showed a mild lymphocytic infiltration. Between

MUCOID INFILTRATIONS IN MYXŒDEMA

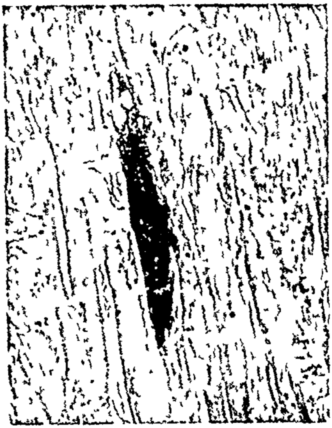

FIG. 1.—Mucoid infiltration of a cardiac muscle fibre. P.A.S. and edicol pea green. Orange filter. × 200.

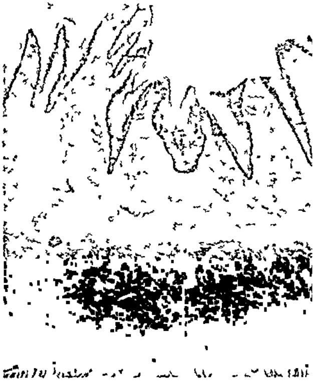

FIG. 2.—A broad band of mucoid infiltrat underlying the epithelium of the tong Toluidine blue. Green filter. × 32.

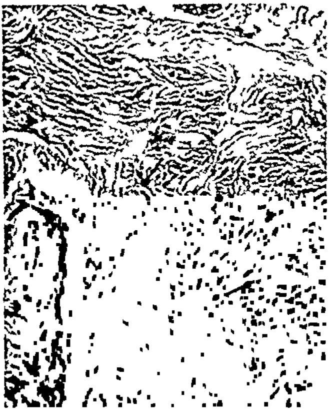

FIG. 3.—The periphery of the area of mucoid infiltration in the tongue. Control section incubated in saline for 24 hours. Toluidine blue. Green filter. × 100.

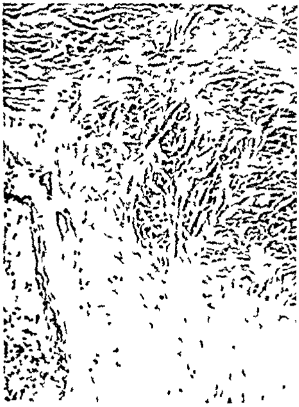

FIG. 4.—An adjacent section to that in fig Incubated with hyaluronidase for hours. Toluidine blue. Green fil × 100.

this layer and the muscles of the tongue there was a broad band of mucoid infiltration lying parallel to the surface (fig. 2). It stained deeply with hæmatoxylin. The muscles of the tongue showed separation of the bundles and of the fibres, but the fibres themselves were normal and showed no mucoid infiltration. The fibres of the *psoas muscle* were widely separated by œdema but were not infiltrated with mucoid substance.

There was moderate atrophy of the *epidermis*. The *superficial dermis* showed a slight diffuse infiltration with a faintly basophilic mucoid substance.

There was no normal *thyroid* tissue. It was replaced by dense fibrous tissue in which were cellular islands made up mainly of densely packed lymphocytes with a few small thyroid acini scattered among them. The *adrenal cortex* was thinned. There was no sharply defined zona glomerulosa and in places the zona fasciculata appeared to extend right up to the capsule. One small encapsulated nodule and several partly encapsulated ones were present in the cortex. Both eosinophil and basophil cells of the *pituitary* were considerably reduced. They were replaced by abnormal large chromophobe cells which were mainly columnar in type. They were closely set and arranged in small solid acini or very occasionally in short, sometimes branched, trabeculæ. Scattered throughout the pars anterior were a number of calcified spherules ; some were laminated and several were surrounded by a ring of abnormal chromophobe cells.

The *seminiferous tubules* were more widely separated than normal and both spermatogenesis and the number of interstitial cells were markedly reduced. There was moderate post-mortem autolysis of the epithelium of the *prostate* but no mucoid degeneration of the muscle fibres.

Mild pseudomelanosis was present in the mucosa of the *colon*. No mucoid infiltration of the submucosa or the muscle coat was found in the colon or the stomach. The *splenic pulp* was congested and there was some hyaline thickening of arterioles. The *lungs* were œdematous with a few small areas of early bronchopneumonia. Only slight centrilobular congestion was seen in the *liver*. In the *kidneys* a few glomeruli were hyalinised and a small amount of albuminous material was present in the proximal convoluted tubules.

## HISTOCHEMICAL OBSERVATIONS

The mucoid infiltrations in the heart and tongue were investigated using the following techniques on paraffin sections :—

(*a*) Staining with Schiff's reagent after oxidation with 0·1 per cent. periodic acid (McManus, 1948). The sections were counterstained with edicol pea-green. This method is referred to as the P.A.S. method.

MUCOID INFILTRATIONS IN MYXŒDEMA

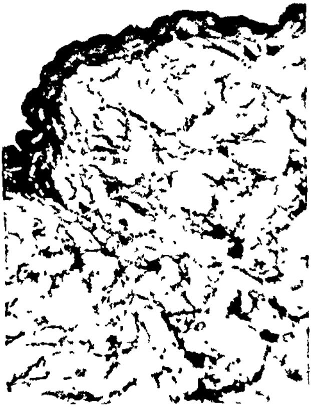

FIG. 5.—Edge of umbilical cord. Control section incubated in saline for 24 hours. Toluidine blue. Green filter. ×400.

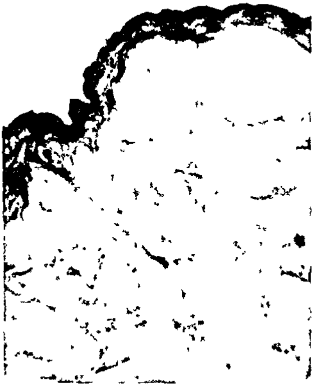

FIG. 6.—Adjacent section to that in fig 5. Incubated with hyaluronidase for 24 hours. Toluidine blue. Green filter. ×400.

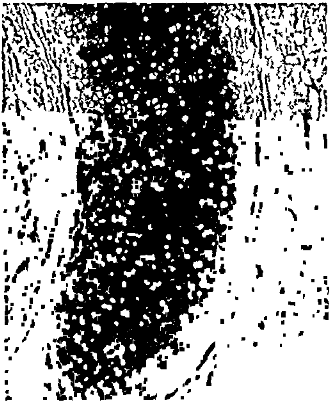

FIG. 7.—Bronchial cartilage. Control section incubated in buffer solution only for 4 hours. Toluidine blue. Magenta filter. ×48.

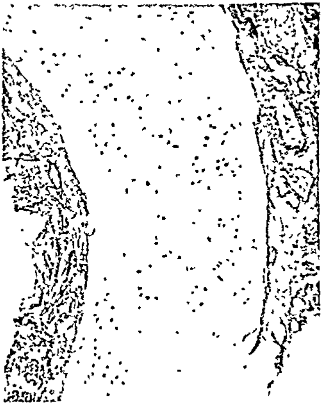

FIG. 8.—Bronchial cartilage. Incubated with diastase for 4 hours. Toluidine blue. Magenta filter. ×48.

hyaluronidase The metachromatic staining of the interstitial substance of the umbilical cord (figs 5 and 6) and of the substantia propria of the cornea was almost completely abolished by incubation with hyaluronidase

Lillie (1949) reported that the strong metachromatic reaction of cartilage was abolished by exposure to the action of the enzyme diastase, but that the cartilage still stained with the P A S method This observation has been confirmed (figs 7 and 8) and it was found that the enzyme did not alter the metachromatic staining properties of the mucoid infiltration in the tongue

The staining reactions of the myxœdematous infiltration and of the normal control tissues are summarised in the table

TABLE

*Staining reactions of myxœdematous and normal tissues*

| | P A S | Meta chromasia with toluidine blue | Most acid *p*H of methylene blue uptake | Effect of hyaluronidase on staining reactions | Effect of diastase on staining reaction |
|---|---|---|---|---|---|
| Umbilical cord interstitial tissue | + | + | *p*H 5 | Metachromasia destroyed | |
| Bronchial cartilage | + | + | *p*H 2 5 | Unchanged | Metachromasia destroyed |
| Bronchial mucin | + | + | *p*H 3 | | |
| *Large bowel mucin* | + | + | *p*H 3 | | |
| Submaxillary gland mucin | + | + | *p*H 5 | | |
| Cornea substantia propria | + | + | *p*H 5 | Metachromasia much reduced | |
| Myxœdematous infiltration in (*a*) Skin | + | + | | | |
| (*b*) Tongue | + | + | *p*H 2 5 | Metachromasia slightly reduced | Metachromasia unchanged |
| (c) Heart | + | — | *p*H 6 | Staining with P A S unchanged | Staining with P A S unchanged |

Sections of the tongue, heart and skin were also stained with Southgate's mucicarmine The infiltrations in the dermis and tongue stained but not those in the heart muscle

Thus there are two distinct histochemical types of mucoid infiltrations in the tissues of this case of myxœdema The mucoid deposit in the muscle fibres of the heart stains slightly with Ehrlich's hæmatoxylin and stains by the P A S method but does not stain metachromatically with toluidine blue and does not take up methylene blue at a more acid *p*H than 5 It still stains with P A S. after exposure to the action of ptyalin of saliva, diastase and hyaluronidase but it gives a negative Feulgen reaction and does not stain with mucicarmine or Sudan black B The deposit in the tongue stains deeply with hæmatoxylin and stains with P A S , it is strongly metachromatic and strongly acid, taking up methylene blue at *p*H 2 5, and the metachromatic staining of the periphery of the infiltration is reduced by hyaluronidase, but unlike cartilage is not affected by diastase. The infiltration of the myocardium is thus a weakly acid mucoprotein, whilst that in the tongue is probably a mixture of hyaluronic acid and a strongly acid polysaccharide resembling chondroitin sulphuric acid.

The deposits in the skin stain with P.A.S., stain metachromatically with toluidine blue and stain with mucicarmine.

## Discussion

### *The nature of the mucoid infiltration and its relation to the œdema*

In 1878 Ord published the results of chemical investigations done for him by Charles on the tissues of a case of myxœdema, showing, he claimed, that there was 50 times more mucin in the skin than in normal skin. This increase in mucin led him to propose the name of myxœdema. Similar results were reported by Victor Horsley (1885) in monkeys rendered myxœdematous by experimental removal of the thyroid.

The Committee on Myxœdema of the Clinical Society of London was unable to find more than a slight increase in the mucin content of the myxœdematous tissues as compared with normal. Since then there has been no further effort to resolve this conflict of view and I could find no record of any modern investigation into the chemical nature of the infiltrations in generalised myxœdema associated with thyroid atrophy. In *pretibial myxœdema*, however, Watson (1946) and Watson and Pearce (1949) found approximately three times as much hyaluronic acid and twice as much chondroitin sulphuric acid in the skin as is normally present.

From the histochemical observations here reported it is likely that the infiltrations in the skin in generalised myxœdema are essentially similar in type to those in pretibial myxœdema. Unfortunately, the present observations on the infiltrations of the dermis were incomplete owing to the small amount of mucoid infiltration present and the inconstant presence of the infiltration from section to section. However, histochemical examination of the skin in myxœdema is of doubtful value as hyaluronic acid is a normal constituent of skin (Wislocki, Bunting and Dempsey, 1947 ; Watson and Pearce, 1949). The tests would almost certainly have to be quantitative.

The swelling of the tissues seen in advanced myxœdema differs from ordinary œdema in several ways. As shown by Byrom (1933-34) there is an increase in extracellular fluid which contains an abnormal accumulation of protein. The swelling does not pit on pressure and at autopsy, as noted by Ord (1878), the swollen and apparently œdematous tissue retains its fluid even when cut up into small pieces. Byrom suggested that the extracellular extravascular accumulation of protein, although of a large molecular weight, exerted sufficient colloid osmotic pressure to oppose that of the plasma proteins and so increased the interstitial fluid, but this theory does not account for the apparent immobility of the increased interstitial fluid It seems probable that the abnormal extravascular protein is conjugated with hyaluronic acid and chondroitin sulphuric acid or some other strongly acid mucopolysaccharide Such conjugates would probably be of too large a molecular weight to exert an effective osmotic pressure but they form hydrophilic suspensoids and are able to attract water very strongly Such water becomes firmly bound, probably by hydrogen bond formation, to the hydrophilic groups in the molecule, especially to the polar groups (Bull, 1943), and so the strongly acid mucoprotein would be especially active If the suspensoid is in the form of a gel this mechanism causing an increase in interstitial fluid accounts for the firm non-pitting nature of the swelling When the mucoprotein is broken down under the influence of thyroxine, as Byrom showed, the interstitial fluid is freed, a rapid diuresis results and the swellings disappear

An analogous condition develops in the swelling of the sexual skin of Rhesus monkeys Here, too, infiltration of the skin contains a mucoprotein which appears and disappears with the œdema Ogston *et al* (1939) considered that the most important factor was the presence in the tissues of fairly high concentrations of protein resembling plasma proteins, but Chain and Duthie (1940) showed that the mucin isolated from the swollen sexual skin of the monkey was broken down by hyaluronidase Recently Duran Reynals *et al* (1950) reported that local collapse of the swollen sexual skin of the monkey resulted from injecting hyaluronidase This suggests that the mucoprotein attracts and retains water and that the mechanism of the swelling of the sexual skin of the monkey is similar to that suggested for the swelling of the tissues in myxœdema

### *The mucoid infiltration of the myocardium*

Since 1918, when Zondek described the clinical entity of "myxœdema heart", there have been many papers describing the histological changes found They include myocardial fibrosis (Higgins, 1936), vacuolation of the muscle fibres (La Due, 1943 , Means, 1948), interstitial œdema (Bastenie, 1944 , Means, 1948) and other unspecified nuclear and cytoplasmic changes, with, in one case, true myxœdematous interstitial infiltration (Bastenie, 1944) Several papers, however, describe mucoid infiltration of the striated and non striated muscle fibres and of the fibres of the myocardium as found in the present case

In 1906 Marchand described basophilic deposits around the nuclei of the muscle fibres Schultz (1921) showed that the mucoid deposits in the muscles of a cretin were not metachromatic and he suggested that they were chromatin that had escaped from the nuclei This suggestion was refuted by Dietrich (1940-41) who described deposits in the myocardium and skeletal muscles of a case of polyglandular dysfunction. He showed that they were Feulgen negative. Foster and Barr (1944) described widespread infiltrations in the muscles of a woman who had suffered from myxœdema for 12 years and suggested that the basophilic material was a form of mucoid. Doerr and Holldack (1947-48) reported a case of myxœdema of some years' standing showing very numerous areas of basophilic degeneration of the myocardium. They suggested that as these areas were so numerous they were to be considered as being the histological characteristic of myxœdema heart.

Basophilic degeneration of muscle fibres has not been produced experimentally in animals by thyroidectomy (Bensen, 1902; Tatum, 1913; Webster and Cooke, 1936) but it has been found by several observers in a large proportion of normal human hearts. Thus Haumeder (1935) found it in 107 of 302 hearts. These findings have been confirmed by Liebegott (1936-37) and Umeda (1940-41). Liebegott found the infiltrations in 21 of 50 hearts and Umeda in 117 of 145 hearts. The deposits occurred without obvious relationship to age or disease. Their relationship to myxœdema was made clear by Fisher and Mulligan (1943) who counted the number of infiltrations in measured areas of hearts from myxœdematous and non-myxœdematous patients. The lesions were present in 67 of 142 control cases; in 56 cases less than 5 fibres being affected, the remaining 11 cases showing an average of 7·7 affected fibres. Eight cases of atrophy of the thyroid showed an average of 40·5 affected fibres. Thus, although the presence of a few areas of basophilic degeneration is of no significance, these areas are extremely numerous in myxœdema, and the great increase in their numbers constitutes the histological characteristic of the myxœdema heart.

## *The changes in other organs*

Ceelen (1921) and Bastenie (1944) described narrowing of the cortex of the adrenal with some loss of the zona glomerulosa and nodule formation in the remaining cortex. These changes are identical with the so-called "senile involution" which the adrenal cortex normally undergoes at about the fiftieth year of life (Aschoff, 1924) and their significance therefore is very doubtful, but they deserve further study as there is a good deal of experimental evidence, reviewed by Zarrow and Money (1949), for a complementary relationship between the thyroid and the adrenal.

Bastenie (1946), in particular, has laid considerable stress on the changes in the colon producing severe constipation and abdominal distension. Although in the present case the colon was distended and elongated, there was no histological evidence of the mucoid infiltration described by him. The reduction in the eosinophil and basophil cells in the pituitary and an increase in the number of chromophobe cells, many of which are abnormally large, agree with the findings of Bastenie The changes in the testes are those previously described in myxœdema (Marine, 1939) but their interpretation is open to question owing to the age of the patient Previous cases with testicular changes have been of about the same age

## SUMMARY

1 A report is given of the post mortem examination of a virtually untreated case of myxœdema

2 Histochemical studies were made of the mucoid infiltrations of the skin, tongue, and myocardium It was concluded that the infiltration in the tongue was probably a mixture of mucoproteins containing hyaluronic acid and chondroitin sulphuric acid whereas the infiltrations of the myocardium consisted of a histochemically distinct mucoprotein

3 The relation of the mucoid infiltrations to the increased interstitial fluid found in myxœdema is discussed and the possibility of binding of water in the form of a hydrophilic suspensoid by the mucoprotein is suggested

4 The pathology of "myxœdema heart" and its relation to the so called basophilic degeneration of the myocardium is discussed

5 The findings in the other organs are briefly considered

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# THE MAST CELLS IN URTICARIA PIGMENTOSA

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(Plates XCI and XCII)

The modern view that the mast cells secrete heparin (Holmgren and Wilander, 1937, Wilander, 1938) has been strongly supported by recent work on the mast cells in various animal species (Oliver, Bloom and Mangieri, 1947, Paff, Bloom and Reilly, 1947, Julen, Snellman and Sylven, 1949-50) as well as in man (Ehrich, Seifter, Alburn and Begany, 1949) and is now widely accepted (Jorpes, 1947)

Much remains to be learnt however, about the actual mechanism of secretion, and for the study of this problem the skin lesions of urticaria pigmentosa provide excellent material They are composed of tumour like collections of mast cells (Unna, 1896), which have been regarded either as congenital anomalies akin to naevi (Little, 1906, Neisser, quoted by Little, Engman in discussion of Hartzell, 1917, Heimann, 1917, Sezary, 1936) or as foci of a mast cell reticulosis (Cazal, 1942) In either event the individual mast cells are indistinguishable from the normal variety, and may be expected to display a normal activity perhaps accelerated by the disease process (Schafer, 1927) The accessibility of the skin lesions, the visible changes they undergo, and the ease with which these may be controlled are additional advantages to commend the use of this tissue

The present study of a typical case of urticaria pigmentosa has been undertaken both to confirm that the mast cells in man function as "heparinocytes" (Ehrstrom, quoted by Jorpes, 1946) and to investigate the process of secretion For this purpose the train of events which follows mechanical irritation of the lesions has been studied in serial biopsies with the aid of histochemical methods capable of demonstrating compounds of heparin type

## Materials and methods

A preliminary experiment was first carried out to determine the staining reactions of mast cells in normal tissues to the histochemical methods which it was proposed to use in the main investigation Both human and rat tissues were examined The human tissues selected were tongue, where mast cells are normally plentiful (Williams, 1900), and œsophagus, where they can be identified readily by their proximity to the large veins in the submucosa The specimens were removed within two hours after death to ensure good preservation of the mast cells, fixed overnight in 80 per cent. alcohol and embedded in paraffin. The sections were stained first by the periodic acid-Schiff's reagent method (McManus, 1946; Hotchkiss, 1948) and the stained mast cells counted, after which the coverslips were removed, alcoholic toluidine blue applied as a counterstain and the mast cells counted a second time. The results are shown in the table.

The rat tissue chosen for examination was obtained from the prepuce, known to be rich in mast cells (Montagna and Noback, 1946), and was removed from a healthy adult female rat immediately after death by exsanguination under ether anæsthesia in the course of another experiment. The specimen was treated in the same way as the previous tissues and the result is illustrated in figs. 1 and 2.

The case of urticaria pigmentosa was a typical example of the disease in a boy aged twelve. Biopsy specimens of the characteristic pigmented macules were taken during an active phase of the disease when general dermographism was present and the swelling of the lesions themselves could be provoked with ease. The local anæsthetic (1 per cent. Novocaine) was injected well away from, and deeply beneath, the selected lesions. The first specimen was an unstimulated lesion, the second a lesion which had been stimulated by firm stroking with a blunt point until full development of the swelling had occurred. The tissue blocks were fixed in 80 per cent. alcohol and embedded in paraffin as before. Deparaffinised sections were stained with toluidine blue, 1 per cent. in 1 : 40 alcohol (Sylvén, 1941), with the periodic acid-Schiff's reagent method (P.A.S.) and with the routine hæmotoxylin and eosin stain.

The special staining methods were selected because of their ability to demonstrate compounds of the heparin type (acid mucopolysaccharides) and the following brief account explains the rationale of their use.

*Periodic acid-Schiff's reagent method* (*P.A.S.*). Mucopolysaccharides possessing two adjacent hydroxyl groups can be oxidised by periodic acid, yielding an aldehyde which is capable of reducing leukofuchsin to a red dye (McManus). Heparin does not always give the reaction owing to its variable chemical structure in which the parent compound, an ester formed by sulphuric acid and a complex mucopolysaccharide (Jorpes and Bergström, 1936), may exist at different levels of esterification. The most potent, that is the most highly esterified form of heparin, is incapable of reacting, since all its hydroxyl groups are occupied by sulphuric acid radicles (Jorpes, Werner and Åberg, 1948). Only the lowest member of the series, heparin monosulphate, has the appropriate chemical configuration for staining with the P.A.S. method (Jorpes and Gardell, 1948) by which it may thus be identified provided that controls are used to detect glycogen and hyaluronic acid, the only substances which in alcohol-fixed tissues, are likely to be present in sufficient concentration to give rise to confusion (Hotchkiss). In the present study, therefore, alternate sections were treated as controls by incubating them for an hour at 37° C. in aqueous solutions of (1) the glycogenolytic enzyme diastasum (B.P.C.), (2) commercial hyaluronidase ("hyalase", Benger). Further control sections were treated by Schiff's reagent alone, without previous oxidation by periodic acid, in order to detect false reactions due to the presence of unsuspected aldehydes.

*Toluidine blue.* Toluidine blue is a member of the group of basic dyes first used by Ehrlich (1877) for specific staining of the mast cells, to the granules of which it imparts a distinctive metachromatic tint. The colour reaction depends upon the acidic property of heparin and is due to the predominance at low *p*H levels, of a tautomeric form which differs in colour from the parent dye. The phenomenon is specific to the extent that mucopolysaccharides of heparin type are the most strongly acid of naturally occurring substances and hence give the most intense colour reaction (Lison, 1935).

## Results

### *The staining of human mast cells by toluidine blue and by the P A S method*

The figures recorded in the table represent the number of mast cells counted after each staining procedure, in the connective tissue occupying the narrow zone between the epithelium and the underlying muscle In the biopsy specimen the comparison was based upon the numbers counted in several of the clumps of mast cells in the subcutis

Table

*The staining of human mast cells by toluidine blue and by the P A S method*

| Case | Diagnosis | Organ | P.A S | Toluidine blue |
|---|---|---|---|---|
| 1 Male aged 55 | Comparatively sudden death lobar pneumonia | Tongue<br>Œsophagus | 43<br>65 | 155<br>127 |
| 2 Female aged 29 | Sudden death obstetric shock | Tongue<br>Œsophagus | 210<br>73 | 377<br>194 |
| 3 Female aged 26 | Sudden death coronary thrombosis | Tongue<br>Œsophagus | 28<br>86 | 60<br>211 |
| 4 Urticaria pigmentosa | Biopsy | Affected skin | equal numbers of mast cells | |

### *Urticaria pigmentosa*

*Unstimulated lesion* The sections show the classical histological features of urticaria pigmentosa, described originally by Thin (1877) and subsequently confirmed and elaborated by numerous authors (notably Unna, 1896 , Richter, 1930-31 , Scolari, 1934 , Graham, Schwartz and Foot, 1942)

The mast cells are arranged in densely packed clumps and columns which surround small blood vessels and hair follicles at the level of the middle and lower cutis Other mast cells penetrate between the acini of nearby sweat glands, accompany the sweat ducts, and form loose connections between all the larger aggregations The individual mast cells are fairly uniform in size with an average diameter of about 20 microns and are rounded except when distorted by mutual compression into more or less angular shapes They possess small round or oval darkly stained nuclei with dust like chromatin and one or two larger nucleoli There are occasional bi nucleated cells and others with bilobed nuclei No nuclei show mitotic activity In alcohol-fixed sections the characteristic granules of the mast cells are not stained by the routine hæmatoxylin and eosin method, and the cytoplasm, no longer obscured by innumerable granules, appears as a tenuous reticulum feebly stained by eosin. The granules, however, are easily demonstrated by toluidine blue and appear as uniform, rounded, closely set bodies filling the cells and obscuring the nucleus. In size the granules resemble those of the eosinophil leucocyte. With toluidine blue the granules also show vivid metachromasia, staining a rich reddish-purple colour which is in marked contrast to the pale blue of surrounding tissues. In sections stained by the P.A.S. method (fig. 3) all the mast cells take the stain and the granules are bright red. Control sections after incubation in solutions of diastasum (BPC) and " hyalase " (Benger) show no diminution of metachromasia in staining when toluidine blue is subsequently applied. In the control section treated by Schiff's aldehyde reagent alone the mast cells fail to stain.

*Stimulated lesion.* Sections stained by hæmatoxylin and eosin show certain morphological changes (fig. 4) ; the mast-cell clumps are smaller and more compact than those of the previous biopsy, and the nuclei, many of which have become spindle-shaped or irregular, are set close together as though the cells themselves had shrunk. A few eosinophils are present in the lesions, one or two in each mast-cell clump with others in and around blood vessels. The connective tissue throughout the section is mildly œdematous.

In toluidine-blue preparations the form and distribution of the metachromatic material is greatly altered (figs. 5 and 6). Most of the mast-cell clumps now show patches almost free from metachromasia, where the cytoplasm of the mast cells is practically unstained and the nuclei are dark blue. In areas which are still strongly metachromatic, the stainable substance of the mast cells seldom appears in the usual granular form but is largely dispersed in sheets, branching filaments and irregular particles.

Sections stained by the P.A.S. method (fig. 7) also show histochemical differences from the previous biopsy. The majority of the mast cells now stain faintly pink, although a few in each clump are still bright red. Generally speaking, these strongly stained cells are the only ones to give the metachromatic reaction when toluidine blue is applied as a counterstain.

### *Subsidiary experiment*

The association of mast-cell changes with tissue œdema, observed in the stimulated lesion, suggested a comparison with other œdematous tissues. Accordingly, fresh human material from the following sources was selected for examination after alcohol fixation and staining with toluidine blue.

1. Specimens of breast carcinoma after mastectomy. Cases were selected in which the subcutaneous tissue was obviously œdematous ; several showed the " peau d'orange " caused by lymphatic blockage and all gave histological evidence of congestion and œdema. 2. A

MAST CELLS IN URTICARIA PIGMENTOSA

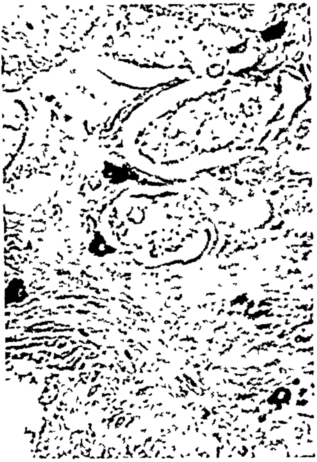

FIG. 1.—Rat prepuce, alcohol fixation, P.A.S. stain. ×400.

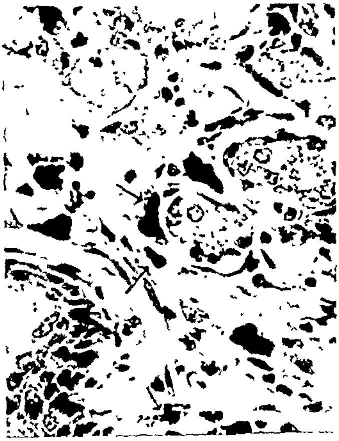

FIG. 2.—The section shown in fig. 1, counterstained by toluidine blue. Mast cells previously unstained are now revealed. ×400.

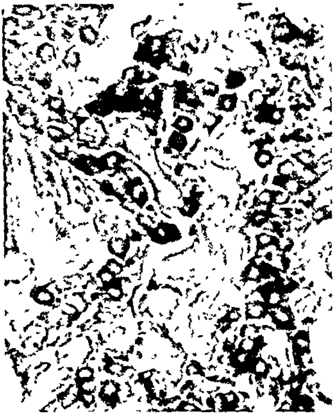

FIG. 3.—Quiescent lesion of urticaria pigmentosa. Alcohol fixation, P.A.S. stain. All the mast cells have taken the stain. ×400.

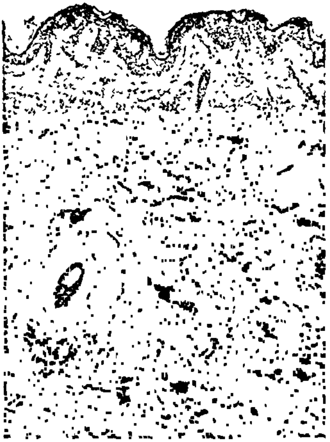

FIG. 4.—Active lesion of urticaria pigmentosa. In the mast-cell clumps the crowded nuclei represent mast cells in the "discharged" state and the spaced-out nuclei others which have not yet released their secretion. Alcohol fixation. Hæmatoxylin and eosin. ×60.

MAST CELLS IN URTICARIA PIGMENTOSA

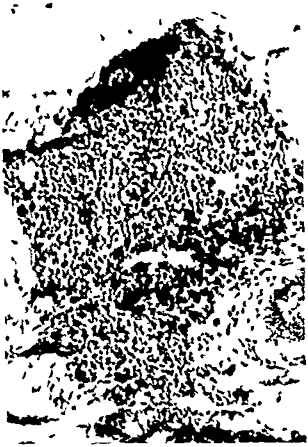

FIG. 5.—Active lesion of urticaria pigmentosa. A clump of mast cells many of which are in the discharged state: only the nuclei are visible and the cytoplasm no longer contains stainable material. Alcohol fixation. Toluidine blue. ×200.

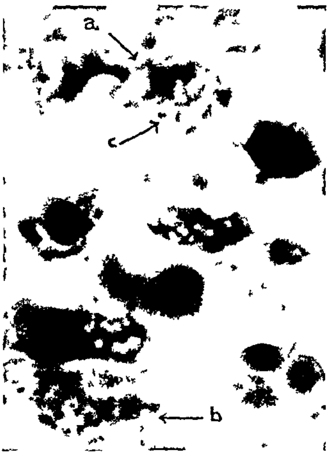

FIG. 6.—Active lesion of urticaria pigmentosa. The dark rounded nuclei represent discharged mast cells. The other mast cells show some of the features of activity

blue. ×1000.

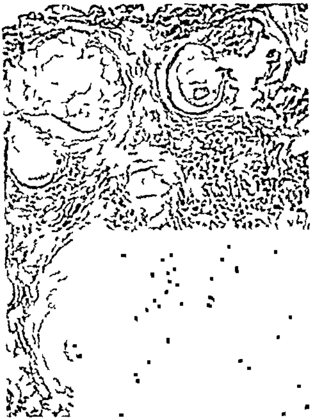

FIG. 7.—Active lesion of urticaria pigmentosa. A group of discharged mast cells scarcely stained. Alcohol fixation. P.A.S. stain. ×200.

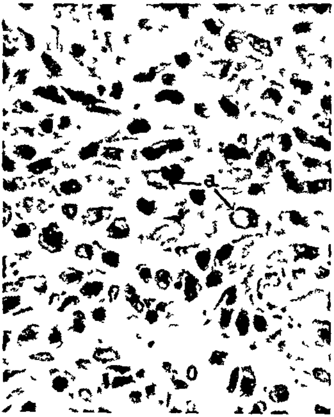

FIG. 8.—Active lesion of urticaria pigmentosa. The polymorphism of the mast cells is well shown. Granulation is lacking and two of the mast cells contain large vacuoles indicated by arrows. Alcohol fixation. Toluidine blue. ×400.

chronic breast abscess, removed by mastectomy. 3. Amputated leg. Amputation had been performed at mid-thigh level for arteriosclerotic gangrene of the foot. The specimen was taken from the region of the knee where subcutaneous œdema had developed immediately before operation.

Examination of the specimens showed that in all areas of acute œdema the mast cells showed changes identical with those in the sections from the stimulated lesion. Frozen sections of breast carcinoma stained by Scharlach R. using watery toluidine blue as a counterstain showed that the vacuoles in the mast cells did not contain lipoid material.

## Discussion

The appearances observed in the biopsy specimens from the present case of urticaria pigmentosa are more easily assessed if the preliminary and subsidiary experiments are first discussed.

The preliminary experiment confirmed previous reports of the staining of mast cell granules by the P.A.S. method in the normal tissues of the rat (Jorpes, Werner and Aberg, 1948) and man (Pearse, 1949) but showed that only a proportion of the mast cells reacted in this way whereas the granules in all of them stained metachromatically with toluidine blue. The interpretation of this finding in conjunction with controls carried out at the same time is that normal mast cells vary in the type of heparin they contain. A proportion of mast cells contain the potent, highly esterified form of heparin, the remainder a less developed variety, heparin monosulphate. It is assumed that mast cells of the second type are less mature than the others.

In urticaria pigmentosa all the mast cells were immature by these criteria, and it is concluded that the continual stimulation to which the lesions are subjected had prevented full maturation of the normal secretion product.

The subsidiary experiment gave an indication of the conditions in which mast-cell secretion may occur by demonstrating that in the presence of acute œdema the mast cells of certain human tissues undergo changes identical with those seen in the biopsy specimens. Since trauma and urticaria were not among the causes of œdema in the control series it follows that these factors cannot be directly responsible for the mast-cell changes in urticaria pigmentosa. The presence of œdema fluid, common to both tissues seems, therefore, to be the immediate stimulus to the mast cells.

The regulation of mast-cell activity in normal tissues is not yet understood but if these cells are to fulfil the role of " heparinocytes " the ebb and flow of capillary filtration would serve as an ideal vehicle for the transmission of heparin to the blood.

The remarkable feature about the vacuolation and disruption of cytoplasm which have been described is the rapidity with which these events occur. The change resembles that of hydropic degeneration except that hydration to bursting point develops within a few minutes of exposure to an isotonic solution, namely, œdema fluid. The explanation of this unusual occurrence may perhaps lie in the fact that the mast-cell granules are composed of mucopolysaccharides, substances which, although feebly osmotic (Hammarsten, 1924; Jorpes, 1947), could rupture the cell by their ability, as hydrophilic colloids to absorb water and increase rapidly in bulk (Bensley, 1934; Day, 1949). The amorphous gel so formed would replace the regular rounded granules of the quiescent mast cell, and it is precisely this transformation which has been observed. It may therefore be concluded that the changes occurring in the mast cells following mechanical irritation of the lesions of urticaria pigmentosa are consistent with a mass discharge of heparin; furthermore, it appears likely that the heparin, by absorbing water, procures its own release from the cells.

For the identification of the mast cells, morphological appearances alone are sometimes insufficient (Westphal, 1880, quoted by Michels, 1938). This is particularly true of urticaria pigmentosa where the mast cells may be discovered at any phase of their accelerated cycle of function with its associated changes of form. In this disease also, as the present study shows, the mast cells may lose even their ability to stain metachromatically with toluidine blue, a property previously regarded as certain proof of their identity (Maximow, 1913). It is not surprising, therefore, if the mast cells in the lesions of urticaria pigmentosa sometimes escape recognition, nor does it seem improbable that distorted and shrunken mast cells have been mistaken for lymphocytes, plasma cells, fibroblasts and the ubiquitous "mononuclears" (fig. 8). The possibility of confusion with cells of other types makes it difficult to assess reports in the literature of scanty mast cells (Hartzell, 1917, case 2; Michelson, 1922), absence of mast cells (Sutton, 1917; Ormsby, 1917, quoted by Sutton; Biach and Kerl, quoted by Hartzell, absence of mast cells at one biopsy with reappearance on a later occasion (Hartzell, 1917; Wise, in discussion of Mount, 1926) or the presence of various adventitious cells (many references quoted by Schäfer, 1927-28; Jadassohn, 1932-33).

The identification of eosinophils alone of the adventitious cells may be accepted without reserve, since their striking bright red granulation always serves to distinguish them from altered mast cells. Apart from their presence in the stimulated lesions of urticaria pigmentosa (this case; Jadassohn, 1932-33; Scolari, 1934) eosinophils have been reported in the stroma of mast-cell tumours of the horse (Sabrazès and Lafon, 1908) and dog (Bloom, 1942). The relationship thus appears significant.

## SUMMARY

1. The lesions of urticaria pigmentosa provide ideal material for the study of the mast cells in man.

2. The application of histochemical methods to serial biopsy

specimens showed that all the mast cells in a case of urticaria pigmentosa contained heparin monosulphate, which they rapidly lost after stimulation of the lesions

3 A proportion of mast cells in the normal tissues of man and the rat contain heparin at a higher level of esterification than the monosulphate and it is therefore concluded that, in the skin lesions of urticaria pigmentosa, full development of heparin is prevented by the continual stimulation to which the mast cells are subjected

4 The changes which accompany secretion in the mast cells are described in detail Watery vacuolation and disruption of the cytoplasm are accompanied *pari passu* by transformation of the characteristic mast-cell granules into an amorphous form

5 In other human tissues, changes identical with those described above develop in mast cells in the presence of acute œdema Taking into account the clinical evidence, it is concluded that in urticaria pigmentosa local tissue œdema provides the stimulus for mast cell secretion

6 The variety of appearances which the mast cells in urticaria pigmentosa present at different stages of activity may conceal their identity or cause them to be mistaken for other cells

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578 67

# SHORT ARTICLES

## RAPID SECTIONS OF THIN MEMBRANES

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In the course of some work on the cultivation of influenza virus in surviving tissue suspensions (Fulton and Armitage, 1951), it was necessary to prepare for histological study sections of chick chorio allantoic membrane As sections from many small pieces of membrane were required the usual methods of fixation and embedding proved very laborious and an attempt was made to discover the simplest procedure which would yield satisfactory results It was observed that immersion of the membranes in dioxan for very short periods adequately fixed, dehydrated and cleared them, and had the great advantage of making the membranes rigid and easy to embed flat Szarski (1937) has already described the use of dioxan as a fixative of embryological material, but it is believed that the techniques to be described here possess several novel features

### *Basic method*

A piece of chick chorio allantoic membrane about 12×12 mm is rinsed in saline and laid out flat in a dry watch glass The membrane is covered with a few drops of dioxan (diethylene dioxide) Care should be taken to avoid inhaling too much of the toxic dioxan vapour After 1 2 minutes the dioxan is removed with a pasteur pipette and replaced by about 2 ml of fresh dioxan If necessary, the membrane is carefully detached from the glass so that it floats freely It is left soaking for 5 minutes The times are not critical The dioxan is pipetted off and replaced by about 2 ml of fresh dioxan It is left another 5 minutes Meanwhile a cellophane cup is prepared by cutting a circle of cellophane 55 mm in diameter and moulding it inside a glass ring, 25 mm in diameter and 10 mm deep The circumference of the cellophane is then bent back over the rim of the glass ring and kept in place with a rubber band By now the membrane in the dioxan will have become rigid It is lifted out of the watch glass by grasping one corner with forceps, carefully blotted and laid flat on the bottom of the cellophane cup It is important not to transfer too much dioxan and the membrane should appear dry

Without any delay the cup is transferred to a vacuum embedding oven maintained at 59° C, and the cup is filled with melted paraffin wax (melting point 52° C) to a depth of about 3 mm The pressure is reduced to 8 cm of mercury After a few minutes the pressure is allowed to rise slowly to atmospheric pressure the total time in the hot wax being about 5 minutes

The cup is now removed from the embedding oven and floated on cold water, after the wax has set, the cup is submerged in the water and allowed to cool for 5 minutes The block is removed from the cellophane cup and trimmed to a square about 15×15 mm The thickness of the block will be 3 mm and one of the thin faces is sectioned, in the usual way, on a microtome Individual 6 μ sections are flattened by floating them on a drop of 40 per cent alcohol on a slide placed on a warm-plate maintained at 50° C. Plain water should not be used for flattening the sections as they tend to tear out of the wax. If the alcohol concentration is increased to 70 per cent. the sections fail to flatten. Excess fluid is removed with a cloth and the sections are dried in a 37° C. incubator. After 10 minutes or so they are ready for staining.

Although it is probable that many staining methods may be used with success, Lillie's azure-eosin technique (1948) has been found very satisfactory. To obtain a good colour balance it is necessary, after bringing the sections to water, and before staining, to mordant them in 1·0 per cent. buffered formalin for 2-3 minutes.

The success of the method depends mainly on the quality of the dioxan. The suitability of a batch of dioxan may be readily assessed by observing the staining of the chick erythrocytes in the sections. With a good batch the cytoplasm of the erythrocytes stains pale pink by the azure-eosin method and the nuclear detail is clearly visible. With inferior grades the cytoplasm of the erythrocytes stains a brick red and nuclear detail is obscured.

The technical grades of dioxan are useless for the purpose, and many of the analytical grades have not proved satisfactory. Uniformly good results have been obtained with fresh batches of *Dioxan puriss* (supplied by L. Light & Co., Colnbrook, Bucks, England). However, the very finest results have been obtained with two batches of highly purified dioxan which had been in the laboratory for some years ; therefore it may be that some ripening process may be desirable in addition to high initial purity.

### *Modified method*

Chorio-allantoic membranes fixed by dioxan are delicate and tear rather easily during section. A more robust membrane is obtained by a small modification which has the further advantages that the stained sections are more familiar in appearance and that the purity of the dioxan is not so critical. For this modified method all the batches of dioxan of analytical grade have proved equally satisfactory. The only change is in the first step of the basic method.

Just before use, a fixative is prepared by saturating methyl alcohol at room temperature with potassium dichromate, and adding neutral 10 per cent. formol-saline to a final concentration of 10 per cent. (0·4 per cent. formaldehyde). The membrane is covered with a few drops of the fixative. After 1-2 minutes the fixative is removed with a pasteur pipette and replaced by about 2 ml. of fresh fixative. If necessary the membrane is carefully detached from the glass so that it floats freely. It is left soaking for 5 minutes.

The fixative is pipetted off and replaced by about 2 ml. of dioxan. If the membrane floats it should be submerged. After 5 minutes proceed as in the basic method, and thereafter the technique is the same except that the sections are not mordanted with formalin before staining.

### *Other applications*

Sections of chick amnion are readily prepared in the same way. Yolk sac is more difficult, but good sections may be obtained if a thin part of the membrane is selected, the yolk is very carefully washed away and particular care is taken to lay the membrane flat. It is also possible to cut good sections of chick embryo intestine and trachea, but the method is not suitable for solid organs.

### *Summary*

Two simple and rapid methods of preparing sections of chick chorio-allantoic membranes for histological study are described. The first method, in which dioxan is used both as a fixative and as a dehydrating agent, demands dioxan

of a very high grade of purity. The second method, in which the membrane is first immersed in a methyl-alcohol fixative, is the method of choice when a satisfactory batch of dioxan cannot be obtained.

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# UROBILINOGEN EXCRETION IN THE FÆCES OF RATS WITH INTESTINAL CULS DE SAC

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Watson, Cameron and Witts (1948) described the development of macrocytic anæmia in rats in which a cul de sac was made in the small intestine, and later Aitken, Badenoch and Spray (1950) showed that the anæmia was usually accompanied by steatorrhœa, although some of the animals became anæmic at a time when they were able to absorb fat normally.

In many of the rats, once the anæmia had become evident, it developed rapidly. The sharp fall in the level of hæmoglobin and the persistent reticulocytosis in the absence of gross bleeding suggested excessive hæmolysis, and the dark colour of the fæces of the anæmic animals lent support to this view. Therefore, as a test for excessive hæmolysis, we decided to measure the output of urobilinogen in the fæces of a group of normal rats and to compare the results with those found in a group of rats suffering from the anæmia.

### *Methods*

The rats were placed in metal metabolism cages over glass funnels with devices for separating urine and fæces. The anæmic rats and the normal controls were as nearly as possible of the same age and size, and all the animals were handled in the same way. The fæces were allowed to accumulate in the receivers for four days and the total quantity passed during this period was used for the estimation. Whenever possible, the fæces of each rat were collected for three consecutive periods of four days, but not all the anæmic rats survived long enough for this to be done.

The anæmia usually proved rapidly fatal, but in some animals it ran a remittent course. Thus, anæmic rat no. 8 (table II) was placed in the metabolism cage while its hæmoglobin was within the normal range (13.15 g. per 100 ml.), although it had been anæmic just prior to the start of the experimental period. During the first four days, the level of hæmoglobin fell precipitately and the excretion of urobilinogen in the fæces rose to a high level. Conversely, anæmic rat no. 3 (table II) improved while under observation and there was a parallel fall in the excretion of urobilinogen.

The urobilinogen in the fæces was measured by a modification of the method of Schwartz, Sborov and Watson (1944). The total amount of urobilinogen excreted in the fæces in each four-day period was recorded in milligrammes

The fæcal output of urobilinogen was also expressed as the hæmolytic index (Miller, Singer and Dameshek, 1942). This relates the fæcal urobilinogen to the degree of anæmia and gives information about the amount of urobilinogen excreted daily per 100 g. of circulating hæmoglobin. The blood-volume of the rats was derived from the figure given by Creskoff, Fitz-Hugh and Farris (1949) of 6·7 ml. per 100 g. of body weight, and the total hæmoglobin was calculated from the values for blood volume and hæmoglobin percentage. The hæmolytic index is the average daily output of fæcal urobilinogen in milligrammes, multiplied by 100 and divided by the total hæmoglobin in grammes.

## *Results*

Table I shows that there was a considerable variation in the amounts of urobilinogen excreted in the fæces of the normal rats in the four-day periods, the mean being 0·38 mg., and the range being 0·10-1·03 mg. This could not be correlated with the size of the fæcal samples. The fæces always contained some animal hair and a small quantity of uneaten food, which must have increased

TABLE I

*Total amount of urobilinogen excreted in the fæces of normal rats in each four-day period and the degree of hæmolysis as expressed by the hæmolytic index*

| Rat no. | Hb. (g. per 100 ml.) at the beginning of the period | Hb. (g. per 100 ml.) at the end of the period | Mean Hb. | Total urobilinogen excreted in fæces in mg. | Hæmolytic index |
|---|---|---|---|---|---|
| 1 | 13·0 | 13·3 | 13·15 | 0·70 | 8·4 |
| | 13·3 | 14·05 | 13·7 | 0·85 | 9·0 |
| 2 | 12·6 | 12·2 | 12·4 | 0·57 | 7·5 |
| | 12·2 | 12·4 | 12·3 | 0·62 | 8·1 |
| 3 | 14·5 | 12·9 | 13·7 | 0·18 | 2·9 |
| | 12·9 | 13·9 | 13·4 | 0·41 | 6·8 |
| | 13·9 | 13·9 | 13·9 | 0·12 | 1·6 |
| 4 | 14·2 | 14·8 | 14·5 | 0·23 | 3·3 |
| | 14·8 | 14·6 | 14·7 | 0·24 | 3·2 |
| | 14·6 | 13·8 | 14·2 | 0·71 | 10·9 |
| 5 | 13·4 | 14·6 | 14·0 | 0·11 | 1·4 |
| | 14·6 | 12·9 | 13·75 | 0·45 | 6·2 |
| | 12·9 | 14·3 | 13·6 | 0·23 | 3·2 |
| 6 | 14·5 | 13·3 | 13·9 | 0·38 | 6·5 |
| | 13·3 | 14·2 | 13·75 | 0·11 | 1·9 |
| | 14·2 | 13·5 | 13·85 | 0·48 | 7·8 |
| 7 | 13·8 | 14·6 | 14·2 | 1·03 | 11·6 |
| | 14·6 | 14·9 | 14·75 | 0·48 | 5·7 |
| | 14·9 | 14·4 | 14·65 | 0·11 | 1·4 |
| 8 | 15·3 | 15·5 | 15·4 | 0·24 | 3·0 |
| | 15·5 | 13·9 | 14·7 | 0·35 | 4·5 |
| | 13·9 | 14·3 | 14·1 | 0·10 | 1·4 |
| 9 | 15·2 | 15·3 | 15·25 | 0·15 | 1·7 |
| | 15·3 | 13·5 | 14·4 | 0·23 | 3·1 |
| | 13·5 | 15·7 | 14·65 | 0·49 | 6·5 |
| Mean | ... | ... | ... | 0·383 | 5·10 |

the error of the measurement when the stools were weighed, but probably did not affect the measurement of the output of urobilinogen. There was no difference in the size of the stools in the two groups. In normal rats the mean weight was 2 62 g and the range 1 02 5·17 g. In the anæmic rats the mean

TABLE II

*Total amount of urobilinogen excreted in the fæces of anæmic rats in each four-day period and the degree of hæmolysis as expressed by the hæmolytic index*

| Rat no | Hb (g per 100 ml) at beginning of the period | Hb (g per 100 ml) at the end of the period | Mean Hb | Total urobilinogen excreted in fæces in mg | Hæmolytic index |
|---|---|---|---|---|---|
| 1 | 11 45 | 11 0 | 11 2 | 0 57 | 8 4 |
| | 11 0 | 13 1 | 11 55 | 0 83 | 5 5 |
| 2 | 8 0 | 10 3 | 9 15 | 1 54 | 42 5 |
| | 10 3 | 8 8 | 9 55 | 1 05 | 31 4 |
| | 8 8 | 10 6 | 9 7 | 0 21 | 6 0 |
| 3 | 4 7 | 4 2 | 4 45 | 6 08 | 364 0 |
| | 4 2 | 9 4 | 6 8 | 3 25 | 104 0 |
| | 9 4 | 11 2 | 10 3 | 1 96 | 37 1 |
| 4 | 8 2 | 8 0 | 8 1 | 0 77 | 14 7 |
| | 8 0 | 9 2 | 8 6 | 0 35 | 6 4 |
| | 9 2 | 9 3 | 9 25 | 0 42 | 6 9 |
| 5 | 8 7 | 7 9 | 8 3 | 1 08 | 21 6 |
| | 7 9 | 7 4 | 7 65 | 0 86 | 18 7 |
| | 7 4 | 8 7 | 8 05 | 0 53 | 11 3 |
| 6 | 9 7 | 9 8 | 9 75 | 3 16 | 59 0 |
| | 9 8 | 9 1 | 9 45 | 2 19 | 43 8 |
| | 9 1 | 10 0 | 9 55 | 0 38 | 7 1 |
| 7 | 11 0 | 6 8 | 8 9 | 1 39 | 38 0 |
| | 6 8 | 8 0 | 7 4 | 0 23 | 9 8 |
| | 8 0 | 9 6 | 8 8 | 0 36 | 9 9 |
| 8 | 15 0 | 4 0 | 9 5 | 1 04 | 33 0 |
| | 4 0 | 5 3 | 4 65 | 0 59 | 32 4 |
| 9 | 8 7 | 3 9 | 6 3 | 0 70 | 30 8 |
| 10 | 9 0 | 9 7 | 9 35 | 0 34 | 7 14 |
| Mean | | | | 1 25 | 39 56 |

TABLE III

*Excretion of urobilinogen in fæces and hæmolytic indices over four day periods*

| Rats | Urobilinogen excretion (mg) | | | Hæmolytic indices | | |
|---|---|---|---|---|---|---|
| | Mean | Difference of means | Probability | Mean | Difference of means | Probability |
| Normal | 0 383 | 0 862 | <0 01 | 5 10 | 35 7 | <0 05 |
| Anæmic | 1 25 | | | 39 56 | | |

weight was 2·86 g., and the range 1·67-4·67 g. None of the animals suffered from diarrhœa and the collections were complete in every case. A comparison of tables I and II shows that the excretion of urobilinogen in the fæces of the anæmic rats is significantly greater than that in the normal animals and that the difference between the hæmolytic indices in the two groups is significant (table III). If the figures are used as a crude measure of hæmolysis, they suggest that the rate of breakdown of blood is increased at least fourfold in the anæmic rats.

## *Summary*

The macrocytic anæmia which develops in rats in which a cul-de-sac has been made in the small intestine appears to be hæmolytic in type. The excretion of urobilinogen in the fæces of the affected animals is increased.

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# ABSENCE OF BARTONELLA INFECTION IN RATS WITH INTESTINAL CULS-DE-SAC AND MACROCYTIC ANÆMIA

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If a blind loop is made in the small intestine of rats a proportion of animals prepared in this way will develop a macrocytic anæmia (Cameron, Watson and Witts, 1949). The precise mechanism inducing this anæmia is unknown and because latent Bartonella infection is common in laboratory rats the results may be criticised on the grounds that the intestinal operation may simply have activated an infection with *B. muris*. In the ordinary way activation of Bartonella infection is seen only after splenectomy but Wills and Mehta (1930-31) have shown that dietary deficiency may also activate the infection. The blood films of our rats with macrocytic anæmia did not show the frank infection with Bartonella which is seen after splenectomy in susceptible animals, when a large proportion of the red cells are obviously parasitised. However, it is difficult to distinguish small numbers of Bartonellæ in the blood, as punctate basophilia, platelets superimposed on red cells and stain precipitates cause confusion. It was not always possible, therefore, to say that a given film was free of Bartonella.

To meet this criticism some further experiments have been made Firstly, anæmic rats have been treated with neoarsphenamine, which is known to cure Bartonella infection in rats (Mayer, Borchardt and Kikuth, 1927), to see whether it influenced the experimental anæmia Secondly, a stock of rats has been kept free of Bartonella infection to see whether anæmia developed in the usual way after operation in these rats

The results obtained with the therapeutic trial of neoarsphenamine are shown in the table As anæmic rats were found, alternate animals were treated with weekly injections of neoarsphenamine or left untreated In the table, survival periods are from the time of identifying the anæmia The results do not suggest that neoarsphenamine prolonged the life of the anæmic animals In both the treated and untreated series there were some rats which had one or more remissions and remained alive for considerable periods, but the treated rats showed no advantage in this respect Nor did neoarsphenamine have any specific effect on the anæmia , for example, treated rat no 8, which remained alive for 230 days after anæmia was first observed, continued to have a fluctuating anæmia despite prolonged treatment with neoarsphenamine

TABLE

*Survival time in days of anæmic rats treated with neoarsphenamine*

| Number | Untreated rats | Treated rats |
|---|---|---|
| 1 | 4 | 4 |
| 2 | 5 | 4 |
| 3 | 8 | 6 |
| 4 | 10 | 7 |
| 5 | 18 | 9 |
| 6 | 28 | 28 |
| 7 | 35 | 46 |
| 8 | 67 | 230 |
| 9 | 70 | |

For the second part of this experiment all rats in stock which might have been infected with Bartonella were destroyed or moved to another room, and all fresh stocks were obtained from a Bartonella free source (Ministry of Supply Animal Farm, Porton) As each batch of new rats was received a sample was splenectomised to ensure that they were free from Bartonella ; none of the splenectomised animals developed the infection As a further precaution all new rats which were not splenectomised were given 10 mg of neoarsphenamine by injection No animals other than Bartonella free rats were allowed in the rat room Throughout the period of these experiments splenectomised rats were kept mixed with the experimental animals, and it was hoped that if Bartonella infection should get into the stock, those animals would act as indicators of its presence None of them developed Bartonella infection Finally, at the end of the experimental period, some of the oldest animals with intestinal loops were splenectomised and these did not develop Bartonella infection It is therefore unlikely that any Bartonella infection was present in the colony in the period under consideration

Macrocytic anæmia did, in fact, continue to appear in experimental rats from this Bartonella free stock

During the period of observation 213 rats were prepared by operation and of these 22 developed an anæmia similar to that reported before Some operated rats remain alive and may still develop anæmia The frequency of anæmia is less than that found earlier but throughout the work on this experimental anæmia, the incidence in operated rats has varied considerably for no obvious reason. The anæmia observed in Bartonella-free rats does not differ in character from that already described though we have the impression that these rats may live longer in the anæmic state.

From these two sets of observations it appears that the development of macrocytic anæmia in rats with blind intestinal loops is unrelated to the presence of latent Bartonella infection in these rats, though it is possible that latent infection may modify the course of anæmia.

## *Summary*

The macrocytic anæmia developing in rats with artificial blind loops in the intestine is not cured by neoarsphenamine, which is effective in Bartonella infection, and the anæmia will develop in rats which are free from Bartonella.

The expenses of this investigation were in part defrayed by the Medical Research Council.

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# OBITUARY NOTICE OF DECEASED MEMBER

## Philip Noel Panton

**Born 26th December 1877. Died 27th December 1950**

(Plate XCIII)

Sir Philip Panton's retirement in 1949 from the post of Consultant Adviser in Pathology to the Ministry of Health ended a career in clinical pathology in which he became pre-eminent. This was not in any way the result of personal ambition, for no one was less imbued with such a motive. Nor could it be attributed to scientific attainment, though in this respect he was by no means unsuccessful. Rather was it the possession of the qualities of leadership, used so unstintingly for the betterment of hospital pathology, that won for him this unique position and incidentally the admiration and affection of his colleagues. In doing this he drove himself hard, particularly in the last ten years, and there was already an indication when he came to retire that his health had suffered in consequence. Unhappily this proved to be true. He was never able really to enjoy his well-earned retirement, and on 27th December 1950, the day after his seventy-third birthday, he died at his home at Bourne End after a short illness.

Philip Noel Panton was born on 26th December 1877 in the small country town of Wareham in the county of Dorset. His father, John Panton, who came of a long line of yeoman farmers, was a brewer; he owned and managed the brewery which stood next to their house on the outskirts of the town on the banks of the river Frome. His mother was a daughter of the famous artist William Frith, one of whose pictures, Derby Day, probably the best known, hangs in the National Gallery. There were five children, three sons and two daughters, a small family for those days. Life was easy for the well-to-do in that spacious Victorian period, and it was in this pleasant atmosphere that the young Philip spent his first eight years. One thing alone is worthy of record in connection with this period of his life, his decision to become a doctor. He had reached the age of six and had gone on a visit to a farmer uncle. Whilst there he had the misfortune to fall and break his leg, and the attachment which he formed for the kindly general practitioner who attended him and set the broken femur determined him to become a doctor himself when he grew up—a decision which his parents did not let him forget. At the age of eight he was sent to preparatory school, first of all to Beckenham Lodge and subsequently to Orley Farm, a preparatory school for Harrow and incidentally the scene of Trollope's novel of that name. In keeping with family tradition, Panton was destined for Harrow and he went on to the big school in 1891 at the age of thirteen, there to spend five years which, from all accounts, were both happy and valuable. Dr Weldon was the headmaster of Harrow in those days and under his guidance the school was enjoying one of its best periods. It was, however, the influence of E. E. Bowen, Panton's house-master at the Grove, which made those five years of such particular value. Bowen was a very considerable personality. He combined a high standard of scholarship in a wide range of subjects with prowess in all forms of athletic sport, and it is easy to understand what an influence for good he exercised on the boys. Panton undoubtedly came under its spell ; the enjoyment which he took in the countryside and in walking was in large part inculcated by Bowen as was, apparently, the impatience which he showed for restrictions which he considered unjustified. Contemporary with Panton at Harrow were several boys who subsequently became famous. G. M. Trevelyan, the historian, was in the same house with him and became Head Boy at the Mount in his time. And L. S. Amery and Winston Churchill were also at Harrow with Panton, the former being Head Boy of the School ; it is interesting that Dr Weldon considered Amery to be much the more brilliant of the two. It must also be recorded that it was whilst at school that Panton received his first appointment as a clinical pathologist ! This was at the Royal Sussex Hospital, Brighton, a quite unofficial post held in vacation time during his last year at Harrow. His " laboratory " consisted of a table on one of the landings and the investigations were confined to the simplest routine urine analysis.

Ever since the early resolve to become a doctor Panton had from time to time doubted his suitability for such a calling. He confessed that he shrank from sick and mutilated people, but not having the moral courage to go back on his original choice he allowed his parents to hold him to it, and in 1896, at the age of 18, he went to Trinity College, Cambridge, there to read for the Natural Science Tripos with a medical qualification as his ultimate goal. Incidentally he came to regard medicine as the finest of all possible careers. At Cambridge he seems thoroughly to have enjoyed himself. He worked sufficiently hard to make a satisfactory showing in examinations. He played football for the Trinity Harrovians in term time and with the Old Harrovians in the vacation. He played cricket of the village-green type occasionally, made many friends and generally entered into the undergraduate social life of the University, in short he took full advantage of the educational opportunities which our two ancient universities alone can provide. And having taken the Natural Science Tripos and obtained the 2nd M.B. he went to St Thomas's Hospital for the clinical part of his training, leaving no debts behind him, in fact he records that his bank balance was 2s. 6d. on the right side ! Here Panton continued his unchequered undergraduate career and in 1903, three years after leaving Cambridge, he obtained the conjoint diplomas of the English Colleges, taking the M.B., B.Chir. of Cambridge a year later. Resident appointments followed, first of all a house physicianship at St Thomas's and then back to Cambridge to hold the post of house surgeon at Addenbrooke's Hospital. His account of conditions at this latter hospital reads strangely these days and he likens the change from St Thomas's to Addenbrooke's to transferring from a Cunard liner to a tramp steamer. Sterile dressings were unknown in the wards and he had to improvise dressing tins out of biscuit tins to make good the deficiency, and the senior surgeon could not do a simple appendicectomy. Panton hastens to say, however, that this was a very temporary, if unfortunate, phase through which this hospital was going, a state of affairs which could not possibly recur to-day. At what particular stage in his career Panton decided to specialise in pathology I do not know, but by the time his appointment at Addenbrooke's terminated the choice had evidently been made and in 1905 he returned to work at St Thomas's in Dudgeon's department, having been awarded a Louis Jenner Research Scholarship. Two of his contemporaries at St Thomas's, A. E. Boycott and H. R. Dean, made the same choice and like him came to occupy distinguished positions in pathology though, unlike Panton, they chose academic careers. The late W. W. C. Topley was another of Dudgeon's students to specialise in pathology and earn great distinction ; he was junior to Panton, coming to St Thomas's when Panton had already started his post-graduate specialisation. And there were others whom Dudgeon inspired, Pulvertaft, Bamforth and Oliver come to mind ; he must have been a great teacher.

Panton's election to the Gibson Research Scholarship of the Society of Apothecaries enabled him to continue the study of pathology with Dudgeon until 1908 when he was appointed Assistant Director of the Clinical Laboratory at St Thomas's. This post was held for only a short time, barely a year, and in 1909 he was invited to join the staff of the London Hospital as Director of the Clinical Laboratory, a post he was to hold with distinction for thirty-seven years. When Panton went to the London the hospital laboratory, then known as the Chemical Pathology Laboratory, consisted of two small rooms in the charge of a technician. It had been started largely at the instigation of the late Mr Frank Kidd and its relation to existing departments was still ill defined. An excellent service in morbid anatomy and histology was provided by Turnbull's department, later to become the Bernhard Baron Institute of Pathology, and the hospital bacteriology was done by Bulloch's department in the Medical College far removed from the wards. Panton thought this latter arrangement was bad since it impeded ready consultations between laboratory man and clinician, so the service which he organised in the hospital, renamed by him the Clinical Laboratory, included bacteriology. This brought him into conflict with Bulloch, and although at first feelings ran high, thanks to Panton's good sense and diplomacy the relations between the Bacteriological Department and the Clinical Laboratory became more tolerant. And when I came to the London in 1926, although some of the hospital bacteriology was still done by Bulloch and his colleagues, the major part came to Panton's department. The transfer of the Bacteriological Department to the hospital had even been discussed, and although this project, warmly supported by the hospital's chairman, Lord Knutsford, had reached the stage of planning, for one reason or another it did not then progress beyond that stage. The idea, however, appealed to Panton. He had always felt that the laboratories doing the hospital pathology should be as near to the wards as possible, not only to make them readily accessible to the clinician but also to encourage the pathologist to go into the wards. From the beginning, Panton had been imbued with this idea, and when in 1934 I succeeded Bulloch it seemed that the opportunity for a further step in this direction had arrived. I was then working in the Clinical Laboratory and Panton suggested that the department in the College which I inherited should move to me in the hospital rather than that I should be banished to the cloistered seclusion of the top floor of the College. Since I shared Panton's view equally strongly and the hospital authorities were agreeable, the move was planned and had it not been for the outbreak of war the work would have started in 1939. Fortunately this proved to be no more than a postponement and although it was not achieved before Panton's retirement he lived to see its realisation. Now, thanks in very large measure to his vision and untiring energy, a Division of Pathology exists at the London which is not merely a spiritual concept.

It is unusual for anyone engaged in a busy practice of hospital pathology to find the leisure for fundamental research; their contributions to advancement of knowledge usually result from observations made in the course of their routine duties. In this respect Panton was no exception and it must be remembered that his appointment at the London, though a salaried one, was only part-time and he had to practise outside as a consultant pathologist in order to live. Although this outside work never grew to a big practice it occupied a not inconsiderable part of his working day and this explains to a large extent the somewhat meagre list of scientific papers published by Panton. It was not lack of ability for research, though as Valentine has pointed out, he had a certain impatience for the irksome detail inevitable in practically any research. However, with Tidy and also with Valentine he recorded observations on the leukæmic states, and he and Sequeira investigated erythrodermia, showing that one form of this condition was associated with a lymphoblastic blood picture. Intrigued by Besredka's work on the relative insusceptibility of the guinea-pig and rabbit to the anthrax bacillus inoculated by other

than the dermal route, he made a study of this phenomenon with Benians, providing a partial confirmation of Besredka's claim and extending the work to the relative susceptibility of the different tissues of the mouse and rabbit to the pneumococcus Perhaps his most important research was on *Staphylococcus pyogenes* and its toxins In this work, done in conjunction with Valentine, the importance of leucocidin in pathogenicity and of its antibody in staphylococcal immunity was emphasised, a conclusion which, though warranted, was at variance with the findings of others And although this work has lost its practical significance with the advent of antibiotic therapy, it still constitutes a definite addition to our knowledge of the natural history of the staphylococcus Any account of Panton's scientific writings would be incomplete without a reference to his textbook on clinical pathology First published in 1913 with Panton as sole author, this popular book has gone through five editions Marrack collaborated with him in the preparation of the subsequent editions and they were joined by May in rewriting the fifth edition which appeared in 1945 , a sixth edition is in the press The fact that this book has gone through five editions is sufficient evidence of its usefulness

Although Panton did not do much original work and perhaps would not even have made a very successful research worker, he was keenly alive to the importance of research, and saw clearly the lines along which progress would come Realising that the work of the Clinical Laboratory was constantly throwing up research problems which he and his staff had not the time to investigate, he sought the means to establish full time research posts in his department In this he was successful, thanks to the generosity of his friend the Rev M S Courtauld who provided a capital sum the income from which sufficed to establish, in 1926, two Freedom Research Fellowships and a Freedom Research Studentship in the Clinical Laboratory Sir Howard Florey and I were the first two Fellows appointed and L F Hewitt held the first Studentship and although Florey left after a year to take up a research post at Cambridge I continued to hold my appointment in the Clinical Laboratory for eight years, a most interesting and enjoyable time

It would have been difficult to find a more delightful and stimulating chief than Panton He allowed complete freedom in the choice of lines of work and did all in his power to provide the necessary facilities , he was sympathetic over the failures and took as much pleasure in the successes as though they had been his own Since those days quite a number of men have worked at the London as Freedom Fellows—J O W Bland, A W Downie, S T Cowan, S D Elliott and J O R Morris—and although to-day, under the National Health Service, the Freedom Fund no longer exists as such, having been merged with Free Funds of the hospital, the Freedom Fellowships have not been allowed to lapse They continue to provide excellent opportunities for the aspirant in medical research and stand as a memorial to the wisdom of Panton and the generosity of his friend Courtauld.

Although Panton's achievements at the London had earned him the high regard of his colleagues there, little was heard of them outside, where he remained comparatively unknown. This was understandable since he avoided scientific meetings and conferences, and as we have seen he had published few scientific papers. It was, however, the work which he did for hospital pathology during the recent war and the early post-war years of reconstruction which enabled pathologists up and down the country to know Panton and appreciate his great qualities. After Munich, when plans were being made for the evacuation of the big centres of population together with their hospital services in case of war, Panton played his part in these preparations and he took the initiative in having prepared a complete register of pathologists and laboratory technicians which was to prove invaluable. With the outbreak of war the London, except for a small residual nucleus, was dispersed throughout Sectors I and II of the London Region, and Panton and I became responsible for the laboratory services of these areas, his being Sector II comprising parts of Middlesex and Hertfordshire. Little existed in the way of laboratories in the hospitals to which the London staff and equipment had been moved and Panton busied himself making good these deficiencies and organising a laboratory service in his sector. He remained here for only a short time, however. The need for someone at the Ministry of Health who could advise on problems concerned with hospital pathology had become urgent and Panton was chosen to do this. For a while he divided his time between the Ministry and his Sector, but the increasing needs of the Ministry soon made his advisership a full-time post. What he had seen of the laboratory services, or rather the lack of them, in Hertfordshire and Essex, convinced him of the urgent need to acquaint himself with the state of things in other parts of the country and to rectify, as far as was possible in wartime, the deficiencies which his experience told him must exist. No one was better fitted for this work than Panton. His earlier experience had shown him not only what an important part good clinical pathology played in maintaining a high standard of medicine, but also how best to organise such a service, and he was determined to see that every hospital in the country possessed these advantages. Convinced that this could only be achieved by establishing personal contact with pathologists and learning first hand of their problems, Panton travelled all over the country, often in considerable discomfort, visiting in the course of these journeys some two hundred hospitals in England and Wales. He saw for himself what was happening, discussed the local problems with the pathologists and hospital authorities concerned, and suggested ways in which the laboratory services could be improved. He met with many difficulties, some of which to lesser men would have appeared insuperable, but Panton usually won through. He succeeded where others might have failed, not only because of his zeal and determination, but because of the great authority with which he spoke, an authority born of vast experience, and, not least, because of the diplomatic way in which he handled these matters In time he came to know personally every pathologist in the country and many of the technicians as well, wherever he went he won trust, admiration and affection His periodic visits were awaited with pleasure He had become the valued friend as well as adviser, and not infrequently, when the business of the day had been disposed of, he would be consulted on personal matters At the centre he examined critically all plans for new laboratories, he attended the meetings of the Committee of Sector Pathologists of the London Region and he arranged for the Regional Advisers in Pathology to meet him periodically in London so that he might be kept in touch with all their problems He was also concerned, in conjunction with the M R C and the services, with arrangements for the training of pathologists and, when trained, with seeing that they were equitably distributed between the armed services, the E M S, the M R C and the University departments It would be difficult to exaggerate the importance of all that Panton did during his term of office at the Ministry He raised hospital pathology throughout the country to a level it had never previously enjoyed and the pathologist to a status of equality with his consultant colleagues And when in July 1948 the National Health Service came into being, so well had Panton planned, the nucleus of an efficient service of hospital pathology existed in every region What is more, when it had become evident that the Public Health Laboratory Service was to be an independent service and, unlike hospital pathology, under central direction, a divorce of the two laboratory services which Panton accepted with regret and some misgiving, he worked loyally and untiringly for the closest possible co operation between the two Public recognition of these services took the form of a knighthood in 1946, an honour in which Panton's many friends and admirers took great satisfaction, even though he himself looked at it somewhat askance

This work at the Ministry resulted in his having to sit on many committees, a duty which he accepted as inevitable if uncongenial Although not a member of the B M A, he was on several of their important committees, including the Central Emergency Committee, the Central Medical War Committee, the Armed Services Committee and the Postgraduate Education Committee In addition, as I have already mentioned, he attended the meetings of the Committee of Sector Pathologists, which ceased to exist with the advent of the N H S, and he was chairman of the Central Pathological Committee which continues to advise the Ministry on all matters concerned with hospital pathology As a representative of the Ministry, he also served on those professional and technical councils of the Whitley Council concerned with laboratory staff, and he helped the British Standards Institute in the work of some of its committees I have always had some difficulty in assessing Panton's value as a committee man. I have, of course, served with him on several of these committees, as well as on many internal committees of the London, and that he could, and did at times, make valuable contributions I readily admit. But committees bored him and this, I think, deterred him from giving of his best, and as a chairman he was apt to allow a committee to wander unguided. He much preferred informal discussions with the few really interested persons to the more formal committee, and with this view all but the most confirmed committee addicts would agree. Of Panton's connection with scientific societies there is little to say. He had been a member of our Society since 1919 but I never saw him at any of its meetings. He was made an honorary member of the Association of Clinical Pathologists in 1948 and he was elected an Honorary Fellow of the Institute of Medical Laboratory Technicians in April 1946 as a recognition of all that he had done for technicians. But he was neither a member of the B.M.A. nor Fellow of the Royal Society of Medicine, and, like Dudgeon, he avoided scientific meetings, holding them to be of little use.

Thus far I have been concerned with what Panton did and have said little about the man himself, though here and there things have emerged to show something of his make-up. He was essentially English in both character and appearance; in fact I always thought that, with his well-shaped head and good features, clear blue eyes, healthy look and neat but sturdy figure, he personified that very English county, Dorset, from which he came. In character he exemplified at its best the type of man of well-to-do parents in Victorian times who had enjoyed a public school and university education, courteous, upright in his dealings, considerate of others, cultured but making no parade of it, and born and bred to be a leader without fear of responsibility however heavy. Panton was a man of first-class intellect and very considerable personality. His command of English, whether in speaking or writing, was of an unusually high order, yet he failed as a lecturer for the sole reason that he was inaudible except to those near him. This was unfortunate because what he had to say was not only of good substance and well presented, but enlivened by that sense of humour with which he was abundantly endowed. He excelled as a writer. As a student at St Thomas's he edited the *St Thomas's Gazette*; it is reputed that he even wrote it almost entirely himself, and in later years he wrote for the amusement of his two grandsons. His reminiscences of medical life which he began writing on retirement had just reached completion; in fact he was happily engaged in putting the legends to its illustrations on the day of his death. Although he played games and enjoyed them both at school and the University, he was no great athlete. He took a delight in the countryside, was well versed in its lore, and enjoyed walking as a relaxation. Fly fishing was another of his hobbies, but greatest of all was landscape gardening. At work in his beloved garden at Otter's Pool all the worries and tribulations of his life, and he had heavy ones to bear, were forgotten When I was considering whether or not to go to the London, I asked a fellow pathologist what sort of a man Panton was, to be told that he was a delightful person, and that is what I found him to be, a truly delightful person As a chief he was exceptionally kind and understanding and as a companion he was without an equal Some of the memories which I cherish most are of moments spent in his company either at work in his garden, watching cricket at Lord's or in the setting of one of our beautiful county grounds, or on holiday in Cornwall Someone remarked of Panton recently that we do not make men like him in England now, and unfortunately there is truth in this, he was the product of a state of society which no longer exists The social revolution is rapidly destroying that leisured and cultured class from which he sprang, and although we have banished poverty and brought educational facilities within the reach of everyone, it is largely the technically instructed we are producing and not the cultured individual Those who applaud this social change and deride what has been termed the "old school tie" tradition should reflect that men like Panton, who stand for all that is best in this tradition, are the salt of the earth

S P BEDSON

The writer wishes to record his indebtedness to Messrs William Heinemann who permitted him to see the MS of the first four chapters of Sir Philip Panton's memoirs and to make use of the information in the preparation of this notice

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# BOOKS RECEIVED

**Primary carcinoma of the liver**

By CHARLES BERMAN. 1951. London: H. K. Lewis and Co., Ltd. Pp. xvi and 164; 84 text figs. 35s.

This monograph cannot be too warmly commended. It combines the fruits of the author's own careful research on hepatic diseases in the Bantu races with a comprehensive and up-to-date review of the whole subject of carcinoma of the liver. By direct and unambiguous diction and by avoiding verbal padding, Dr Berman has packed into a small volume an astonishingly large amount of information; indeed it contains all that is essential about our knowledge of liver cancer, and it will certainly become the standard work of reference on this disease. The illustrations, most of them photographs or photomicrographs, are uniformly good and most of them excellent. The bibliography contains about 700 references, and the analytical details of these which are scattered throughout the text make it clear that the author is intimately familiar with them and that they are not merely inserted on the time-honoured principle of "swelling the crowd". Berman's own work on Bantu livers convinces him that hepatic cirrhosis and cancer are closely related and that "dietary factors, especially protein deprivation, are among the most important causes of cirrhosis". In view of the great prevalence of cirrhosis and cancer in the Bantu races, he insists that South Africa is the proper place in which to undertake a thorough investigation of these diseases, a proposition the truth of which could have no stronger support than his own admirable research. The book is beautifully produced and the only misprint that a pernickety reviewer could discover was "tumour" in the reference to an American paper.

**The acridines: their preparation, physical, chemical and biological properties and uses**

By ADRIEN ALBERT. 1951. London: Edward Arnold & Co. Pp. xiii and 381; 1 plate and 31 text figs. 70s.

The anti-infective action of basic acridine derivatives first attracted attention when it was reported that trypaflavin (acriflavine), a quaternary derivative synthesised for Ehrlich, acted as a potent chemotherapeutic agent in mice infected with *T. brucei*. Browning with Gilmour, Gulbransen, Kennaway and Thornton, then observed that in high dilutions in culture medium it inhibited and killed various bacteria, both Gram-positive and negative, and that blood serum did not reduce these effects; Shiga independently described its antibacterial property toward *V. choleræ*. The former workers showed too that the corresponding tertiary base, later called proflavine, had similar activity. Proflavine and acriflavine, being relatively little toxic for mammalian tissues as compared with the "general protoplasm poisons" previously used as antibacterials, were then employed as "surface antiseptics" for the prevention and treatment of sepsis in wounds. Albert made a valuable practical contribution by introducing an almost colourless member of the group (5-aminoacridine or aminacrine), which is an extremely potent antiseptic. The aminoacridines have found a wide and continuing clinical use. Although they do not influence general bacterial infections and cannot be administered effectively by the systemic route, their powerful action on various pathogenic bacteria locally in the tissues has been proved beyond question. The antimalarial agent atebrin (mepacrine), which has proved so valuable as a substitute for quinine, also belongs to this group. Important investigations have been carried out by Albert and his colleagues on the relations between physical-chemical properties and antiseptic action among the acridine compounds, and it has been demonstrated that the determining factor is the basic strength of the substances; those which are well ionized (as kations) being highly antiseptic, while those poorly ionized are feeble. It is stressed that the tissues should not be brought into contact with solutions of those salts which have an acid *p*H. The book, in addition to containing a collected account of the extensive original researches of Albert and his associates on the synthetic, physical-chemical and biological aspects, also describes all the significant work which has been done on the acridine series. Thus the successive sections deal with preparative methods, physical and chemical properties, biological properties and other uses—as dyes, antioxidants, industrial disinfectants, microscopic stains, and in one case, luzigenin I, as a striking example of a synthetic chemiluminescent substance; an appendix deals with the manipulation and purification of acridine compounds. There is a list of over 1800 references. Readers of the *Journal* will be specially concerned with the biological part, which occupies about a quarter of the text. The book is the only comprehensive one on the subject and is likely to remain for a long time an authoritative guide to the chemistry and biological properties of the acridine series.

## Color atlas of pathology

Prepared under the auspices of the United States Naval Medical School. 1950. Philadelphia, London, Montreal: J. B. Lippincott Company. Pp. xi and 546; 1053 figs. in colour on 365 plates and 4 text figs. £7.

The pattern of this book can best be conveyed by describing a page selected at random. Page 481, for example, deals with infantile rickets. Down its right half are three pictures, each 3×2½ ins., the top one a radiograph of the forearms, the middle one a coloured large-section photomicrograph (×15) of the growth cartilage and metaphysis, and at the bottom a higher-power view (×50) of the growth line, also in colour. On the left half of the page is a column of text which starts with a brief clinical history of the case illustrated and continues with short legends to each of the three pictures. There is a similar arrangement of each page in what is termed the "color unit", varied only by the choice sometimes of gross-specimen or clinical photographs. The clinical photographs are excellent, so are many of the pictures of gross specimens, but others of these are too small to be useful. It is in these two classes of work that colour photography can be of most value. Photomicrographs seldom gain anything by the use of colour except where specific colour reactions are to be illustrated—the prussian-blue test for iron may be quoted as a simple example. Incidentally, this staining method was introduced by Perls and should not be called either the "Perl reaction" (p. 84) or "Perl's reaction" (p. 373). Sections stained with Congo red for amyloid or with Sudan IV for fat are among those which are successfully shown in this book. But standard hæmatoxylin and eosin sections lose much in detail and gain nothing in contrast when reproduced in colour. Even with the high technical competence of the publishers, there is minute inexactness of register which makes many of the low-power views a slightly hazy mass of little contrasted violaceous tones The reviewer sampled two hundred consecutive photomicrographs, all in colour, and decided 184 of them would have been better in black and white Every pathologist will enjoy seeing beautiful pictures of diseases not usually encountered in this country, such as leprosy and bubonic plague (complete with rat flea), and of such rarities as the reddened palate showing an allergic reaction to ginger ale (p 281)

The text was largely written, it appears, by Dr Charles F Geschickter who around 1944 was Chief of Pathology in the U S Naval Medical School It might have been better to have preserved the anonymity of the title page, for the text is not even as good as the pictures The brief descriptive columns accompanying the photographs are, in general, adequate and factual, although certain unorthodoxies appear here and there *e g* the risky diagnosis of Kaposi s sarcoma in a woman who had had a mastectomy for carcinoma two years earlier (p 243), angiomesothelioma of bone (p 502), and the perpetuated confusion caused by including the megaloblast with the ancestors of the normocyte (p 7) The purpose of the written introductions ( ' descriptive surveys ' ) to each section of the atlas is obscure These oversimplified synopses can hardly serve any useful function in education For instance, twenty four pages of discursive text are made to cover diseases of the alimentary tract from herpes labialis to rectal carcinoma while embracing paragraphs on German measles and balantidiasis

There is no section devoted to the nervous system This important omission is curious since brain and spinal cord tend to reproduce classical morbid appearances more constantly than most organs and therefore provide material well suited to an atlas There are also other large omissions —generative organs, breast and eye, for example But this fact serves only to emphasise how arbitrary the selection of material for this or any other atlas may be

Habits of thought change with changing methods of instruction The cynic may remark that the habit of thinking has long since withered in the student of medicine through disuse and that his intellect has atrophied from the press of too easily assimilated intelligence Gone are the days of the closely printed page with the closely reasoned argument The summary speech of the advertiser displaces the measured tones of the scholar, and the clarity of the monochrome pales before the glories of technicolor The use of atlases is more prevalent in American medical schools than our own Most teachers in this country to day reject the visual memory type of instruction, substituting analytical observation, knowledge of principles and thoughtful reasoning as the basis of skill in pathological diagnosis

## A laboratory guide to the anatomy of the rabbit

By E Horne Craigie 1951 Toronto, University of Toronto Press London, Oxford University Press (Geoffrey Cumberlege) Pp viii and 113, 28 text figs 21s

The author of this book, who is Professor of Comparative Anatomy at Toronto, is well known for his revision of the last two editions of Bensley's *Practical anatomy of the rabbit* Those who know that volume will appreciate the scope of the present book if it is described as the basic one third of " Bensley " modified in such a way as to make a handy dissecting guide for the novice The early chapters are very elementary, but here and throughout the writing is direct and instructive The major part of the book is made up of concise descriptions of the gross anatomy of individual bones muscles, nerves and viscera The book is probably intended for 1st M B or general science students, but it will be a useful acquisition in many pathological laboratories where rabbits are numbered in the establishment. Technicians and animal attendants will find in it a clear and sound introduction to mammalian anatomy and the experimental pathologist may on occasion be glad to have at his elbow this straightforward account of the less as well as the more familiar parts of the rabbit.

## Enterobacteriaceæ

By F. KAUFFMANN. 1951. Copenhagen: Ejnar Munksgaard. Pp. 338. 50s.

This book brings together under one cover a series of studies on Enterobacteriaceæ which have been published in recent years by Dr Kauffmann and his colleagues, and thus presents in convenient form a mass of detailed observations which every worker on this subject will from time to time want to consult. The nomenclature and classification are based on the Report of the Enterobacteriaceæ Sub-Committee of the Nomenclature Committee of the International Association of Microbiologists which was presented to the International Congress on Microbiology in 1950. The author divides the family Enterobacteriaceæ into some 11 overlapping groups. Separate chapters are devoted to 9 of these, the remaining 2, Erwinia and Serratia, receiving only brief mention. The primary differentiation of the groups, and further subdivision within certain groups, is effected by biochemical reactions, but the final identification of types is based on serological tests.

Approximately a third of the book is devoted to the Salmonella group, and the very full account of antigenic structure, variation, and serological technique is of great interst and importance. The views which the author holds on capsular or "K" antigens, which include V Antigen (antigen No. five), Vi Antigen and M Antigen (mucoid antigen) differ in some respects from those which are currently accepted in this country, and merit careful study. A full and lucid account is given of the methods used in preparing antigens and also the antisera used in identifying the formidable list of types now included in the Kauffmann-White Schema.

In the chapter on the Escherichia group, the emphasis is again placed strongly on antigenic pattern, and no attempt is made to subdivide the group in other ways, although biochemical and other characters are fully discussed. Twenty-five main groups are defined by differences in O antigen, and these are further broken down to give 93 types, in addition to which mention is made of many other types which have not yet been fully investigated. Evidence is adduced to show that one person may harbour in his bowel a number of different types at one time, and when re-examined after an interval of months may have a new series including one or two of those present at the first examination.

The organisms comprising the familiar "paracolon" group which lies between Salmonella and Escherichia are split into 3 goups which have been named Arizona, Ballerup and Bethesda. Work on these groups is not completed.

The chapter on Shigella follows the recommendations made by the Shigella Commission, and adopts a classification which agrees closely with one which has been in general use for a number of years. K (capsular) antigens have been found in certain types, and account for some anomalous findings. Most workers with practical experience in bacillary dysentery will agree with the removal of Alkalescens and Dispar from the Shigella group, and the creation of a provisional Alkalescens-Dispar group; in this also several types have been described. In the Klebsiella group (Friedländer, Aerogenes, Aerobacter) O and K antigens serve to define 5 groups and 18 types. Proteus is divided into 3 groups, one of which embraces *P vulgaris* and *P. mirabilis*, and is divided according to the author's classification into 49 "O" groups *P morgani* and *P rettgeri* have not yet been serologically analysed A provisional group named Providence has been created intermediate between Shigella and Proteus

The approach to the classification of the Enterobacteriaceæ made in this book is essentially individualistic, for the author himself has formulated many of the theories relating to the different types of antigen and has devised many of the methods used He and his colleagues have devoted a vast amount of painstaking and meticulous care to making the analyses and compiling the tables which are the basis of the work Yet it is impossible to avoid feeling that serology has been allowed to dominate the picture to a greater extent than is warranted We know that many serological types of Enterobacteriaceæ are stable and relatively permanent and are constantly being isolated the world over But what of the others showing but slight deviations from the better known species which have been found perhaps only once ? Are these permanent types of long standing which will persist into the future, or are they fortuitous and ephemeral variants which in natural surroundings are here to day and gone to morrow ? Time alone will show, but the ever swelling volume of Salmonella types strongly hints at something of this kind, and indeed the author's observations on the changing types of Escherichia in the human intestine could well be explained by the operation of a sort of kaleidoscopic variation If this occurs, elevation of these variants to the dignity of type status is a procedure which can only lead to confusion There appears to be more than a little justification for the adoption of a conservative attitude in this matter

A minor point, quite unrelated to the subject matter, is that in parts of the book the names of groups and types are italicised while elsewhere they are not

## The differentiation of Escherichia and Klebsiella types

By F Kauffmann 1951 Springfield, Ill , Charles C Thomas Oxford , Blackwell Scientific Publications Ltd Pp x and 57 15s

Friedlander's bacillus and *Bacterium aerogenes*, culturally indistinguishable, are combined into one group, the Klebsiella As a group they can easily be distinguished from typical *Bacterium coli* (the Escherichia group) but the intermediate and irregular forms are not mentioned

The greater part of the book deals with the serological analysis of each group which, because of the multiplicity of antigens, is difficult and complex In addition to the usual O and H antigens, strains of the Escherichia group have K antigens that may be thermolabile (L and B) or thermostable (A) , those of the Klebsiella group have only one K antigen of the thermostable capsular type The twenty five Escherichia O groups are subdivided into 93 types by the K and H antigens , but finality has not been achieved for the last chapter tells us that the type common in infantile gastro enteritis (D 433 of Joan Taylor, α of J Smith) forms a new O group named 111 (one hundred and eleven) by Kauffmann

The four O groups of Klebsiella are subdivided by an ever increasing number of capsular antigens, some of which are related to Escherichia A antigens in the way that Friedlander type B is related to pneumococcus type II

This book, which is a companion volume to Kauffmann's *The diagnosis of Salmonella types*, is a simple statement of one worker's methods , there are only ten references, five to Kauffmann and five to other Scandinavian workers It contains much less information than the author's more comprehensive *Enterobacteriaceæ*

## Recent advances in the study of plant viruses

By KENNETH M. SMITH. 2nd ed., 1951. London: J. & A. Churchill, Ltd. Pp. viii and 300; 27 figs. on 20 plates and 33 text figs. 22s. 6d.

Great progress has been made in all branches of virus research since the first edition of this book appeared in 1933, and the present edition is in fact a new book rather than a new edition. The value of this book is somewhat lessened by the fact that its publication follows closely that of another text on the same subject. The subject-matter is divided in 12 chapters dealing with the nature and economic importance of plant viruses, the external and internal symptoms and their variation as influenced by the environment and the effects of viruses on the metabolism of the diseased plant. *Insect transmission is discussed critically and a separate chapter is* concerned with the other modes of transmission. The next four chapters deal with the purification, properties and size of the viruses and the importance of the electron microscope in virus research. The last three chapters are devoted to strains, immunity to infection, serology and control measures. An admirable account of symptomatology and the relation of the viruses to their vectors is given. Chapter VIII, amounting to 59 pages, is devoted to a detailed description of methods which were used in the past to estimate the size of virus particles. Such methods are now only of historical interest and should have been mentioned briefly. The photographs are of high standard and the book contains many references.

## Bakteriologie und Serologie

By LOTHAR HALLMANN. 1950. Stuttgart: George Thieme Verlag. Pp. xi and 644; 374 text figs. (93 in colour). DM. 29.70, $7.

This book is written by a bacteriologist in charge of a hospital laboratory as a guide for those engaged in this type of work. Stress is naturally placed on the routine methods to be followed in establishing diagnosis by isolating and identifying bacteria, skin tests and serological reactions. Fungus, protozoal and virus infections are all treated, though not exhaustively. The serological reactions used in diagnosis are considered at greater length, the description of complement-fixation technique being especially detailed. The sections on general laboratory methods are particularly useful; the preservation of cultures, anaerobic cultivation, counting of bacteria, preparation of agglutinable suspensions, and methods used in relation to the use of antibiotics and such practical aspects are clearly described. In the last section of the book there are summarised notes on specimens to be collected and examinations to be made in the diagnosis of individual infections. Formulæ for stains, fixing solutions, indicators and general reagents are included and there is a useful list of references to recent papers on special culture media.

There are, here and there, a few surprising statements: precipitins are said to be thermolabile bodies (p. 376), the cause of mumps is unknown (p. 323) and mention of the Schick test on p. 216 suggests that the time for reading the results is after 24-28 hours. Some of the newer bacteriological techniques used to aid epidemiological investigations in this country are not to be found in the book, for example phage typing of typhoid and paratyphoid B bacilli, phage or serological typing of staphylococci. There is no mention of the prenasal and postnasal swab for the diagnosis of pertussis in young children and methods for the investigation of virus infections are rather sketchy. On the whole, however, the material is well arranged and this book is full of useful information, the illustrations are good and for routine bacteriological work it should prove a useful and reliable guide to the hospital pathologist

### Exotische Krankheiten und Krankheitsverlaufe

By F O Horing 1950 Stuttgart Georg Thieme Verlag Pp xi and 407 , 52 text figs DM 36, $8 55

Germany has always been proud of her tropical medicine institutes Although circumstances have severely curtailed their scope, they remain in being, and the present textbook is evidence of continued interest in the subject The book covers more than tropical diseases as such Its scope is diseases not indigenous to Germany and also indigenous diseases in so far as they run an altered course in hot climates It is addressed primarily to the doctor practising abroad and to the " stay at home " who sees imported cases of exotic diseases Professor Horing writes from the clinical stand point , hygiene, bacteriology and parasitology are touched upon only so far as is unavoidable Even with this restriction the field is too large for anything like exhaustive treatment within 400 pages There is nothing in the book not easily available in the English literature, with the exception of some unfamiliar and debatable ideas on pathogenesis, but Professor Horing does not here develop his theories in sufficient detail to interest the pathologist The text will present no difficulty to a reader with a competent knowledge of German but is beyond the beginner

### Praktikum der wichtigsten Infektionskrankheiten

By C Hegler 5th ed , revised by H E Bock, 1950 Stuttgart Georg Thieme Verlag Pp xi and 277 DM 14 70, $3 50

This is a short textbook for the general practitioner Although it sets out to cover only the most important fevers, many others, not in themselves of major importance, are described This is either because they are of differential diagnostic interest, *e g* Filatow Duke disease, or, like Q fever and homologous serum jaundice, because information is not readily available in German literature Many advances made in other countries during the war years are even yet not easily accessible to the general practitioner in Germany and Professor Bock explicitly sets out to provide information of this kind The book is fully up to date but contains nothing which cannot be found in a modern English textbook It is lucidly written and a reader with but little German will find no difficulty in following the text

# PROCEEDINGS OF THE PATHOLOGICAL SOCIETY OF GREAT BRITAIN AND IRELAND

*13th and 14th July* 1951

The eighty-third meeting of the Society was held at the University of Liverpool on Friday, 13th July, and Saturday, 14th July 1951

### Communications and demonstrations

Those marked with an asterisk are abstracted below

T. Crawford. Anitschkow cells of the rabbit's heart.

H. A. Sissons and G. Hadfield. The influence of cortisone on the repair of experimental fractures.

*Mary Barber and A. Delaunay. The effect of the plasma of animals treated with cortisone on the growth of macrophages and fibroblasts in tissue culture.

F. R. Magarey and J. Gough. The effect of cortisone on the reaction to silica in the peritoneal cavity.

H. Hamperl. Circulation of blood and localisation of pathological processes in the liver.

J. V. T. Gostling. Observations on the viability of cells of sarcoma 37.

M. A. Epstein. The effects of cold and desiccation on the cells and virus of the Rous sarcoma.

*W. Goldie and D. H. Collins. Erysipelothrix arthritis in rabbits.

Elizabeth M. Harper and G. B. S. Roberts. Experimental actinomycosis in mice.

A. de Minjer. Hypothermia, thyroid gland and hepatic glycogen.

J. B. Duguid and G. S. Anderson. The pathogenesis of hyaline arteriolosclerosis.

A. L. Wells. Arterial changes in coal workers' pneumoconiosis.

Ian Rannie. Observations on the pathogenesis of cholesterol lesions in rabbits' arteries.

A. H. Cruickshank. In-vivo studies of the resistance to infection in alloxan diabetes.

C. V. Harrison. Long survival in Hodgkin's disease.

A. M. Barrett. Hypoplasia of the internal jugular veins associated with aneurysm of the great vein of Galen and cerebral arterio-venous hamartoma.

L. Dmochowski, L. Hoyle and R. D. Passey. Immunological studies of the milk factor.

R. J. O'Connor. The action of acridine compounds on cell division.

*W. St. C. Symmers and D. F. Barrowcliff. Platelet thrombosis syndrome.

J. M. Naftalin and G. H. Cushnie. Pathology of bracken poisoning.

H. E. Hutchison, J. A. Milne, A. Porter and J. B. Rennie. Further experiments with H.P.C.

R. Pirrie. The effect of splenectomy and reticulo-endothelial blockade upon the anæmia of lead poisoning in guinea-pigs.

E. S. Duthie. Mode of action and antigenic properties of coagulase.

S. D. Elek. Some observations on a proteophage.

E. M. Brieger and A. M. Glauert. Reproduction in mycelial strains of avian tubercle bacilli.

Betty Dawson and K Zinnemann The incidence and type distribution of capsulated *H influenzæ* strains in the upper respiratory flora of children during 1948 1950

G M Williamson and K Zinnemann The occurrence of two distinct capsular antigens in *H influenzæ* type e strains

H Proom The classification of *Proteus rettgeri*

C E Kellett Complementary activity of the blood in certain diseases

R H Gorrill and D Hobson The agglutinating property of complement

E N Dafaalla and M A Soltys Studies on agglutination of erythrocytes by pathogenic organisms of the clostridium group, with special reference to *Cl septique*

T H Flewett and C E Challice Host cell virus relationships in fowl plague and influenza infected eggs, a study by electron microscopy of thin sections

G T Stewart The virulence of human tubercle bacilli

*C N Iland, W St C Symmers and A P D Thomson Tuberculosis in the ferret (*Mustela furo* L)

A Macdonald Contagious pustular dermatitis in man

N Wood An interesting if not important epidemiological contribution

J W Czekalowski and G O Horne The isolation of a leptospira from a case of abacterial cystitis

J W Czekalowski and J W McLeod Observations on the appearance of cultures of leptospira in solid media

H B Maitland, F N Marshall, G F Petrie and D T Robinson Some observations on antibacterial diphtheria serum

F N Ghadially and H J Whiteley Mesenchymal tumours of the corium of the goldfish, *Carassius auratus*

J Gough and J E Wentworth Large sections of diseases of the liver mounted on paper and in plastic

W E Kershaw Some specimens of *Loa* sp in monkeys

T H Flewett and C E Challice The cutting of thin sections for electron microscopy

F A Langley, J P Smith and A S Woodcock Debatable uterine tumours

C Raeburn Primary tumours of the heart

A L Woolf Histological demonstration of the passage into the circulation of a thromboplastic substance derived from the myelin in cases showing perivascular demyelination

O C Lloyd Epidermal melanoblasts revealed by DOPA staining

N Wood The winter vomiting disease or epidemic nausea, diarrhœa, etc

## Abstracts

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### EFFECT OF PLASMA OF CORTISONE TREATED GUINEA PIGS ON THE GROWTH OF FIBROBLASTS AND MACROPHAGES IN TISSUE CULTURES *IN VITRO*

*Mary Barber and Albert Delaunay

*From the Pasteur Institute, Paris*

Numerous authors have drawn attention to the retarding effect of cortisone on the formation of granulation tissue *in vivo* Thus Ragan and his colleagues found that the hormone inhibited the formation of granulation tissue in cutaneous wounds and caused a delay in all phases of the healing of fractures (Ragan *et al*,

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1949; Blunt *et al.*, 1950). Taubenhaus and Amromin (1950) and Coste *et al.* (1951) found that cortisone had a similar inhibitory effect on granulation-tissue production in turpentine abscesses in rats. The present investigation was undertaken to determine whether the plasmas of animals treated with cortisone have a direct action on the growth of fibroblasts and macrophages *in vitro*.

Cultures of fibroblasts were prepared from the hearts of chicken embryos; for macrophages guinea-pig spleens were used. Tests were carried out on *initial cultures without a preliminary period of normal growth*. Small fragments, about 1 mm. in diameter, were placed in 1 drop of an extract of guinea-pig spleen and 1 drop of heparinised guinea-pig plasma was added. At least 16 cultures were used for each plasma tested and the growth was compared with that of a similar series of cultures in normal plasma.

The preparation of cortisone used throughout was cortisone acetate (Merck). This was administered to guinea-pigs by three routes: subcutaneous, 1 mg. per 30 g. body weight once daily for 3 days, the animals being bled on the fourth day; intraperitoneal, a single dose of 25 to 5 mg. 1-4 hours before bleeding; intravenous, 25 mg. 1 minute-2 hours before bleeding.

With this large dosage many animals showed signs of exhaustion at the time of bleeding and some even died before blood had been taken. Furthermore, the plasma of a number of animals which were ill contained microorganisms, and other plasmas showed evidence of hyper-coagulability. In all, 20 plasmas suitable for tissue culture work were obtained from treated guinea-pigs.

Of the plasmas of 20 animals treated with cortisone, 11 showed a marked inhibitory action in tissue cultures; of these, 7 inhibited the growth of both *fibroblasts and macrophages and 4 inhibited the growth of fibroblasts* but had no apparent action on spleen cultures. In many instances the inhibition of growth was complete and with other plasmas where growth did occur it was often atypical and showed signs of early degeneration. Of the plasmas of 6 guinea-pigs treated with large doses of de-oxy-corticosterone acetate, 4 showed no significant effect, 1 inhibited the growth of fibroblasts and macrophages and 1 stimulated the growth of both types of cell.

Centrifuged washed deposits of cortisone crystals were resuspended separately *in the plasma of 6 normal guinea-pigs*. After incubation at 37° C. for 2-48 hours the mixtures were centrifuged and the clear supernatant was used as plasma for tissue cultures. In all 6 instances these treated plasmas caused an almost complete inhibition of growth of fibroblast and spleen cultures.

In an attempt to determine whether the inhibitory plasmas from the above experiments were cytotoxic as well as inhibiting growth, 5 such were tested for their effect on the chemotactic movements of polymorphonuclear leucocytes. In 2 instances the movements of leucocytes suspended in these plasmas were normal. The other 3 plasmas caused an almost complete inhibition of chemotaxis, but when the leucocytes were separated by centrifugation and resuspended in normal plasma the power to respond to chemotactic stimuli was restored. *Thus in no instance were the cells killed.*

It is clear from the results here recorded that the plasma of guinea-pigs treated with large doses of cortisone in many instances strongly inhibits the growth of fibroblasts in tissue culture *in vitro* and in some instances that of macrophages also. In view of the large doses used this direct effect cannot be regarded as physiological. Similarly large doses, however, have been used in most of the experimental studies on the inhibition of granulation tissue by cortisone *in vivo*.

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616.72—002—092.9:576.851.5
(*Erysipelothrix rhusiopathiæ*)

## EXPERIMENTAL ERYSIPELOTHRIX ARTHRITIS IN RABBITS

William Goldie and Douglas H. Collins *

*From the Departments of Pathology of St James's Hospital, Leeds, and of the University of Leeds*

After demonstrating the association of *Erysipelothrix rhusiopathiæ* infection with sporadic, naturally occurring polyarthritis in swine, Collins and Goldie (1940) induced a similar arthritis in these animals by intravenous inoculations of Erysipelothrix cultures. Swine receiving multiple injections regularly developed severe arthritis. A milder form of the disease followed single injections. The arthritis was of bacteræmic origin and proliferative and non suppurative in type.

Comparable experiments have now been made with rabbits using intravenous doses of *E. rhusiopathiæ* from 100 to 1000 times smaller than those previously used in demonstrating other pathogenic effects, *e.g.* monocytosis and focal liver necrosis (Barber, 1939). Fifteen rabbits received from 2 to 6 weekly intravenous injections of almost water-clear suspensions of Erysipelothrix (total dose 7-28 million organisms) and 14 developed signs of arthritis (8 killed and examined *post mortem*, 6 still alive). Twelve rabbits received a single intravenous injection of 10 million Erysipelothrix and 10 developed arthritis (5 killed and examined *post mortem*, 5 still alive). Recognition of arthritis in the living animal is difficult and hitherto post mortem examination has tended to reveal a more widespread arthritis than was thought to exist during life. Abnormalities of posture and of gait were the first signs, some of which were demonstrated in a cinematograph film. Fluctuant synovial effusions could often be palpated, and at a later stage the joints were found to be thickened. Stiffness and crepitus could be observed in some of the larger joints in the chronic stage of the disease.

Arthritis was first noticed at intervals ranging from 6 to 35 days after the first injection, and some arthritic animals have now been observed for periods up to 200 days. After death, 12 joints (the three main joints of each limb) were opened and examined in each animal. In 5 of 13 rabbits the disease was monarticular. The largest number of joints simultaneously affected in one animal was 10. Since the easily accessible knee-joints have probably been the only joints commonly inspected at post-mortem examination of rabbits, an important observation was the fact that out of 26 knee joints opened in 13 arthritic animals, only 10 were diseased. Carpus and shoulder were more frequently affected and hip joints very rarely.

Histological examination showed a proliferative type of arthritis with granulomatous enlargement of the synovial villi, multiplication of the synovial-lining cells and increased vascularity of the stratum synoviale with heavy leucocytic infiltration. In most joints, lymphocytes and plasma cells predominated and large lymphoid foci, near the synovial surface or in the villi,

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* In receipt of a grant for expenses from the Medical Research Council

were frequently encountered. But polymorphonuclear heterophile leucocytes were often plentifully diffused through the stratum synoviale, and small pockets of suppuration were occasionally seen. Joint exudates were mucinous and turbid, not usually frankly purulent, and contained 50 to 80 per cent. of granulocytes, the remaining cells being monocytes and lymphocytes. Cartilage destruction or digestion was not seen, and adherent synovial pannus was found only at the margins of the cartilages. A chronic inflammation in the marrow spaces of the bone ends and reactive sub-periosteal osteogenesis were sometimes noted. Arthritic animals are being kept alive with a view to studying late cicatricial changes in the joints. These have not yet been seen.

Blood cultures were occasionally positive during life (3 out of 16), but, in animals coming to necropsy, blood and spleen cultures were consistently negative, with the exception of one animal with bacterial endocarditis. Joint cultures have been positive in 22 of 48 arthritic joints examined after death.

The original strain of *E. rhusiopathiæ* was No. 1224 of the National Collection of Type Cultures. We have not so far been able to detect any variation in the pathogenicity of this strain as the result of rabbit passage.

A verrucose bacterial endocarditis with large vegetations on the mitral valve was found in one animal. Lymphocytic infiltration in the portal tracts was seen in many livers but no parenchymatous necrosis. Light perivascular cuffing by lymphocytes was seen around some small coronary branches in two hearts, but, so far as we know, arthritis was the sole constant major pathogenic effect of *E. rhusiopathiæ* infection under the conditions of our experiments.

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# PLATELET THROMBOSIS SYNDROME

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The so-called platelet thrombosis syndrome (thrombotic thrombocytopenic purpura, thrombocytic acroangiothrombosis) has not attracted much attention outside the United States. We have recently seen the following case.

A previously healthy man, aged 40, developed mild sciatica associated with early sacro-iliac ankylosis. Four months later he developed hæmaturia with granular casts and 0·2 per cent. of protein in the urine, which previously was normal. His blood urea was 146 mg. per 100 ml., Hb. 10·4 g., erythrocytes 3·9 million per c.mm. and blood pressure 160/90. Acute nephritis was diagnosed. For six weeks the patient had intermittent pyrexia with disproportionate tachycardia; hæmorrhagic retinopathy developed and anæmia increased; nitrogen retention, proteinuria and hæmaturia diminished. Transitory mental and neurological disturbances occurred. Examination of the cerebrospinal fluid showed: protein 40 mg. per 100 ml., globulin test (Pandy) faintly positive; cells, sugar and chlorides normal. He was treated by blood transfusion, penicillin and streptomycin. After the streptomycin course severe hallucinosis occurred during four days. Thereafter, blood urea fell rapidly to 38 mg.; proteinuria, macroscopic hæmaturia and fever disappeared; Hb. rose from 7 to 9·2 g.;

tachycardia persisted Improvement continued, and 12 weeks after the initial hæmaturia the patient was sent home A week later he was readmitted with mild purpura Mental disturbance and hæmaturia recurred, blood urea rose to 72 mg and Hb fell rapidly Leucocytes were normal No platelet count was made Total plasma proteins were 8 g per 100 ml (albumin globulin ratio, 4 4) He died in coma, 15 weeks after hæmaturia began

Necropsy revealed multiple petechiæ in the kidneys (230 g each) and heart, the myocardium was pale, renal architecture blurred, spleen and liver slightly enlarged, brain œdematous and marrow hyperplastic

Heart, kidneys, adrenals, liver and spleen were examined histologically Eosinophil thrombi were present in a great many blood vessels in the outer third of the myocardium, in the capsular zone of the adrenals and in the renal cortex Mainly capillaries were affected, but also many arterioles and occasional venules Thrombus so distended some arterioles that their differentiation from venules was difficult Intra hepatic lesions were confined to a few arterioles The spleen was not involved Inflammatory cells were absent from the lesions Recent thrombi were granular, older ones hyaline Some granular thrombi contained occasional blood cells and a little fibrin No iron pigment or hæmatoidin was found, hæmoglobin was demonstrable only in erythrocytes No unequivocal platelets were identified, although many particles were of appropriate size and staining reactions (Romanowsky stains, phosphotungstic acid hæmatoxylin, periodic acid Schiff), the thrombocytic nature of the thrombi was presumed from the absence of a significant proportion of other thrombic constituents Endothelial changes were prominent in many affected vessels Segmental loss of endothelium was associated with adhesion of thrombus to the affected site Endothelium proliferating from intact areas of the affected vessels grew out over the surface of the thrombi and later into their substance Organisation was accompanied by irregular recanalisation and slight juxta vascular fibrosis When cut lengthwise or serially some thrombi were seen to have extended, free in the lumen, from a limited area of attachment to the vessel wall Cross sections of such propagated thrombi often appeared as a hyaline, endothelialised core unattached to the vessel wall in the plane of section

Focal, subendothelial accumulation of hyaline material, the prethrombotic lesion of Gore (1950), was seen in a very few vessels, protrusion of this material into the lumen led to local loss of endothelium and platelet deposition

In the kidneys organisation of intracapillary thrombi caused a peculiar interstitial glomerular sclerosis A few other glomeruli showed "wire loop" changes indistinguishable from those of disseminated lupus erythematosus There was no evidence of ordinary glomerulonephritis Minute, fresh or organising infarcts were present in the myocardium and adrenals Petechiæ had no constant relationship to the thrombotic lesions

The first comprehensive account of this condition was given by Baehr *et al* (1936) The specific vascular abnormality had been described in one previous case (Moschcowitz, 1924, 1925) Up to now 32 cases have been reported, all of them in the American literature, including one described by Pagel (1949) in which the history and pathological findings were typical We are indebted to Dr Pagel for allowing us to study sections from this case and for permission to quote his comment that he agrees that it was probably an example of so called platelet thrombosis Of the 32 cases, 21 have been diagnosed by workers in four centres, suggesting that familiarity with the condition leads to its more frequent recognition

The disease occurs in both sexes and at any age Onset is usually sudden Typically, the illness is characterised by a diagnostic tetrad of fever, purpura, hæmolytic anæmia and multifarious, fluctuating neurological disturbances Death results, usually after 2 4 weeks

Laboratory findings include thrombocytopenia and severe normocytic normochromic anæmia, often with a high reticulocytosis and possibly circulating normoblasts. Leucocytes are usually little altered, but immature cells may be numerous; the picture has sometimes been described as "leukæmoid". Bleeding time is prolonged, clot retraction defective and clotting time usually normal. Excess globulin and moderately raised total protein have been found in the cerebrospinal fluid.

Macroscopic findings at necropsy are not characteristic. Histological examination reveals wide dissemination of the remarkable vascular changes described above.

The ætiology is unknown. Allergy has been suggested, and sensitivity to sulphonamides noted. Relationship to the so-called collagen diseases seems likely. Coexistence of typical lesions of platelet thrombosis, disseminated lupus erythematosus and polyarteritis nodosa has been observed; a family history of the latter diseases has been noted twice in cases of platelet thrombosis syndrome.

The syndrome has twice been correctly diagnosed during life. It should be recognisable oftener. Biopsy might prove diagnostic: muscle biopsy nine weeks before our patient's death was normal. No effective treatment is known. Splenectomy possibly accelerates death. The apparent relationship to the collagen diseases suggests that cortisone or ACTH may prove valuable, particularly if the diagnosis is made early.

We are indebted to Dr S. R. F. Whittaker, Leamington Spa, and Dr V. V. Kalbian, Stratford-on-Avon, for access to the clinical record of the case described here.

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616—002 . 5 : 599 . 742 . 4 (*Mustela furo* L.)

# A NOTE ON TUBERCULOSIS IN THE FERRET (*MUSTELA FURO* L.)

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The ferret is highly susceptible to experimental infection by bovine-type tubercle bacilli but relatively insusceptible to human and avian strains (Dunkin *et al.*, 1929; Goret and Mariette, 1939). Spontaneous tuberculosis is commoner than the few reported observations might suggest (Dunkin *et al.*; Dalling and O'Brien, 1935; Spanedda, 1941). MacIntyre (personal communication) observed that as many as 60 per cent. of ferrets obtained from dealers may be infected with avian tubercle bacilli, which cause an insidious, Yersin type of tuberculosis with splenomegaly, in contrast to the extensive gross lesions typical of the less common infection by bacilli of the bovine type. Infection results from feeding with sick poultry or infected milk or meat, and is preventable. The absence of macroscopically obvious tuberculous lesions in avian-bacillus infection and in atypical cases of infection with bovine strains can lead to unwitting transmission of the disease to other animals in the course of experimental procedures.

We have recognised only one case of spontaneous tuberculosis among over 500 ferrets. A mature female (710 g.) was killed five days after the onset of a rapidly progressive sickness. Turbid exudate in the thoracic and abdominal cavities contained enormous numbers of acid-and-alcohol-fast bacilli. The peritoneal subserosa and the walls of the hollow viscera were thickly infiltrated by firm or caseous yellowish tissue. Fæces and urine contained innumerable acid and alcohol-fast bacilli. The abdominal lymph nodes were considerably enlarged and replaced by similar tissue. The liver was slightly enlarged but otherwise normal. The spleen was slightly enlarged and its pulp studded with nodules resembling miliary tubercles; a similar appearance was present in the bone-marrow. The cut surfaces of the kidneys were normal. The thoracic viscera were relatively little affected. The lungs contained scattered nodules little larger than miliary tubercles. A caseous mass replaced the retrosternal lymph nodes. There were minute vegetations on the aortic-valve cusps and on the intima of the aorta The meninges, brain and spinal cord were normal. The necropsy findings were those of a primarily abdominal tuberculosis with predominantly lymphogenous spread

The main histological features of the macroscopic lesions were bacillosis, histiocytosis and necrosis. Organisms were so numerous, especially towards the periphery of the necrotic areas, that Ziehl-Neelsen preparations appeared red to the unaided eye There was a narrow zone of well-preserved, bacillus-laden histiocytes at the periphery of the necrotic areas. Most of the histiocytes were rounded, and epithelioid metamorphosis was not striking; few contained more than one nucleus, and no Langhans cells were found. Fibrosis was inconspicuous except around the largest lesions There was a diffuse histiocytosis of the spleen and bone-marrow, with multifocal caseation and innumerable bacilli. Small caseous foci were present in the gastro intestinal mucosa. In the liver, bacilli were numerous in the patchy histiocytic accumulations in Glisson's capsule and in nodular foci of Kupffer cell hyperplasia, there was no necrosis. The pulmonary nodules and cardiovascular vegetations consisted of collections of bacillus-laden histiocytes, without necrosis. Intrahistiocytic and free-lying bacilli were found in all parts of the body except the brain and spinal cord. Bacilli were also present in mesothelial cells, in cells lining adrenal and pituitary sinusoids, and occasionally in intravascular leucocytes. Extramedullary hæmopoietic foci were numerous.

A male ferret was infected experimentally with the organism isolated from the spontaneously infected animal. It remained in seemingly excellent condition for six months, and then suddenly became ill and was killed five days later. A fibrous nodule was found at the intramuscular inoculation site. The liver weighed 98 g. (normal maximum, 60 g ). The spleen filled most of the belly and weighed 138 g. (normal maximum, 20 g ), equivalent to 11 per cent. of the body weight (1280 g.). Apart from their size both organs appeared normal. The only other abnormalities were a few small nodules in the lungs. Histologically, there was no necrosis. The splenomegaly was due to the presence of aggregates of large, rounded histiocytes, many of which contained acid-fast bacilli, although seldom more than two or three in a cell. The bone-marrow showed a similar histiocytosis The same process in the portal tracts and throughout the lobules gave the liver an appearance reminiscent of the pattern of infiltration seen in malignant reticuloses in man, bacilli were present in many of the cells. There was only slight histiocytosis in the lymph nodes, where bacilli were scanty. The pulmonary nodules also consisted of collections of histiocytes; acid fast bacilli were more numerous in them than in any other lesions. There were scanty acid-fast bacilli in the scar at the inoculation site.

Only occasional histiocytes, many of them containing one or two bacilli, were found in any of the other tissues.

The organism is a short, strongly acid-and-alcohol-fast bacillus which grows slowly on Dorset's egg medium, Löwenstein-Jensen medium and inspissated ox serum. Its growth is not augmented by adding glycerol to any medium or by incubation at 41° C. Scarcely any yellow pigment is produced, and the growth is flat, dry and confluent. Smears do not show the clumps and twisted coils of bacilli characteristic of smears of most strains of tubercle bacilli; instead, the organisms are spread remarkably evenly with a fairly uniform distribution. The organism is pathogenic to the rabbit (dose of 0·001 mg. intravenously), guinea-pig and ferret, but not to the rat. The results of inoculation of birds are not yet available. Re-isolated from the experimentally infected ferret, the organism showed the same cultural characteristics and pathogenicity as previously.

An interesting feature was that the acid-and-alcohol-fast organisms in smears of exudate and excreta from the original ferret and in sections of its tissues were also strongly Gram positive when stained by the ordinary methods at room temperature. In cultures and in the tissues of animals subsequently infected, including the second ferret, this staining characteristic is not so apparent.

We are indebted to Dr C. D. Cruickshank, Bromsgrove, for looking after the animal which was infected experimentally, and for his help with the bacteriological studies.

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# THE FORMATION OF ABNORMAL DIPHTHERIA ANTITOXIN IN THE EARLY STAGES OF IMMUNISATION

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It has long been known that qualitative differences may exist among diphtheria antitoxic sera, so that their curative power in animal experiments may not be solely dependent upon the antitoxic value as determined by standard methods. It is always possible that sera of low curative power may be deficient in certain unrecognised but necessary antibodies. Nevertheless there is much evidence that the generally recognised antitoxin present in samples of sera from different hyperimmunised horses may vary qualitatively in the efficiency with which it neutralises the appropriate toxin. The term "avidity" was introduced by Kraus to describe this qualitative property (see Kraus and Schwoner, 1908; Kraus and Baecher, 1913).

It is probable that all workers experienced in the comparison of samples of antitoxin by *in vivo* methods have the same general conception of the meaning of avidity. Two factors contributing to this property are the speed of combination of the antitoxin with toxin (Madsen and Schmidt, 1926, 1930) and the firmness of the combination (Glenny and Barr, 1932*a* and *b*). At the present time these properties cannot be fully measured by an in-vitro test. It therefore seems unfortunate that some workers now use the word avidity to describe certain manifestations observed in the titration of other types of antibody by in vitro tests only.

Madsen and Schmidt (1926) found that an Lo mixture (*i.e.* one just non toxic to guinea pigs after subcutaneous injection) of a certain toxin with a fast flocculating antitoxin was non toxic to rabbits when injected intravenously immediately after preparation. A similar mixture prepared with a slow flocculating serum was lethal to rabbits until 14 hours after preparation.

These two sera were further compared by injecting them intravenously into rabbits one hour after the intravenous injection of 6 M.L.D. of toxin. Whereas 10 units of the fast serum were sufficient to save the life of the rabbits, 150 units of the slow serum were needed. These results indicated that speed of flocculation might constitute a useful measure of avidity.

It was suggested by Glenny and Llewellyn-Jones (1931) and Glenny and Barr (1932*a*) that the quality of antitoxin was related to its serum ratio $\frac{\text{in-vivo value}}{\text{in-vitro value}}$ rather than to speed of flocculation; sera of comparable avidity may have widely different flocculation times. Whereas the in-vitro value of an antitoxin can in general be represented by a constant figure, the in-vivo value of a non-avid antitoxin varies according to the conditions of the test and is lower than the in-vitro value when determined under standard conditions.

That mixtures of toxin with non-avid antitoxin might dissociate and become toxic on dilution was shown by Glenny, Pope and Waddington (1925). They found that certain mixtures which produced no reaction in guinea-pigs after intracutaneous injection in the undiluted state, produced toxic reactions if injected after being diluted 1 in 100.

Extending this work, Glenny and Barr (1932*b*) studied the effect of intracutaneous injection into guinea-pigs of different dilutions of the same mixture of toxin and non-avid antitoxin. No reactions were produced by dilutions of 1 in 100,000 or weaker because insufficient toxin was present; nor were reactions caused by dilutions of 1 in 100 or stronger because the toxin was all combined with antitoxin. The injection of dilutions between 1 in 2000 and 1 in 50,000 was followed by the appearance of typical toxin reactions caused by toxin dissociated from combination on dilution *in vitro*. Slightly stronger dilutions, 1 in 200-1 in 1000, produced unusual reactions in the form of rings of varying width with an outer diameter of 12-15 mm. or more. The development of such reactions suggested that further dilution of the mixtures in the tissue fluids, on diffusing out from the site of injection, had brought about dissociation after injection.

Further evidence of dissociation of mixtures *in vivo* was obtained from the results of intravenous injection of different volumes of the same mixture of toxin and non-avid antitoxin into rabbits (Glenny and Barr, 1932*b*). Doses of 1·0 ml. or more were non-lethal to rabbits, but doses between 0·001 and 0·5 ml. caused death. This was ascribed to the liberation of toxin from the mixture on dilution in the circulation of the animals after intravenous injection; some doses had undergone an additional earlier dilution *in vitro*. The dilution of the larger, non-lethal doses was assumed to be insufficient to bring about the dissociation of a fatal dose of toxin. Dissociation can occur on diluting mixtures which have stood for several weeks; this suggests that the combination of toxin with some at least of the non-avid antitoxin is loosely linked and does not become firmer with time.

A quantitative measure for use in the comparison of avidity of different samples of diphtheria antitoxin was introduced by Glenny and Barr (1932*a*). Their procedure consists in the titration by the guinea-pig intracutaneous method of antitoxin at two levels of dilution. The volume of antitoxin necessary to neutralise the Lr dose of toxin in a total volume of 2 ml. is divided by that required to neutralise to the same end-point in a volume of 200 ml., and the resulting figure is termed the "dilution ratio". The antitoxins of which a greater volume is necessary for neutralisation in the 200 ml. test are relatively non-avid and have a dilution ratio less than 1·0. In the titration of good quality antitoxins, on the other hand, the same or a slightly lower volume of serum is required to neutralise the test dose of toxin in the more dilute test. The actual value obtained for the dilution ratio depends upon the toxin used in titration (Barr, 1949), the more toxic the toxin, in terms of the number of

M.R.D. per Lr dose, the lower the dilution ratio. The dilution ratio of most sera, however, when referred to that of a suitable chosen standard antitoxin, was found to be constant within the limits of error, whatever toxin was used.

## EXPERIMENTAL

It is probably known to most observers engaged in testing the antigenic efficiency of diphtheria prophylactics, that non-avid antitoxin is frequently produced by the test guinea-pigs. This was first pointed out by Glenny, Barr, Ross and Stevens (1932), and recently Jerne and Maaloe (1949) using a modification of the dilution-ratio test of Glenny and Barr (1932*a*) obtained lower titres for such sera when tested at a high dilution. The first example of non-avid antitoxin-production in guinea-pigs was recorded by Glenny (1913-14) before the significance of the finding was understood. Antitoxin of extreme non-avidity is often present in the sera of guinea-pigs one month after a small single injection of purified toxoid adsorbed on to mineral carriers. It is not possible to measure the dilution ratio of such antitoxin because the titre is too low, but rough tests on pooled samples have shown that more than three times as much antitoxin may be needed for neutralisation if the test mixtures are diluted 10-fold before injection. In the routine titration of such samples, large faint positive reactions may be seen in the guinea-pigs used as indicators, and any attempt at close testing reveals the long range between the nil end-point and that at which a good positive reaction is obtained. These findings are evidence of non-avidity.

The reason for the appearance of this type of antitoxin has not been investigated, and at one time it was supposed at these laboratories that it might be a property of this particular species of animal. Recently, however, evidence of non-avidity has been observed in the titration of certain sera from human subjects bled 14 days after a boosting dose of prophylactic (unpublished). Petrie (1942-44) also found that antitoxin produced by horses after a single dose of tetanus prophylactic tended to show a higher titre when tested by the mouse method than by the guinea-pig method. The lower titre obtained in guinea-pigs was assumed to be due to dissociation of the test mixtures after absorption and dilution in the body fluids, this effect being greater in the larger animal than in the smaller one (see Glenny *et al.*, 1932).

These findings suggested that the quality of antitoxin produced might be connected with a low degree of immunity such as exists in prophylaxis. The quality of diphtheria antitoxin produced in the very early stages of immunisation has now therefore been studied.

### *Materials and methods*

For an investigation of this kind, horses are the most suitable animals to use, because frequent injections can be given and relatively large blood samples taken as required. In the determination of the dilution ratio of samples of low titre, it was necessary to modify the original method and the actual ratio introduced by Glenny and Barr. The ratio used in this work was that of the amounts of serum necessary to neutralise toxin at the Lr/100 and Lr/1000 levels of titration. In practice two toxin dilutions were used, the Lr in 10 ml. and the Lr in 100 ml. Varying amounts of suitably diluted serum were added to 1 ml. of each toxin dilution, the test mixtures were made up to 2 ml. with saline and 0·2 ml. of each injected intracutaneously into guinea-pigs half an hour after preparation. Ring reactions were rare in the titration of the non-avid samples. They are usually produced after the injection of relatively strong mixtures, and in these particular titrations the mixtures were probably insufficiently strong for their formation. The absence of this type of reaction increases the ease of reading the results, and in consequence the accuracy of the values allotted. Titrations were made at 10 per cent. differences at each dilution and repeated 4 or 5 times. It has been found advisable to accept as neutral mixtures those containing the minimal amount of free toxin. By allotting figures to reactions graded according to size and intensity, it is possible to calculate the composition of such mixtures.

## *Results*

In the first experiment, a dose of 5 ml. (250 Lf doses) of diphtheria prophylactic alum-precipitated toxoid (A.P.T.) was injected intramuscularly into a number of so-called normal horses. Blood samples were taken 10 and 14 days later and subsequently at weekly intervals. Some of these horses had low titres of circulating antitoxin before injection and were therefore "naturally immune". Others with no detectable antitoxin before injection responded by measurable antitoxin-production 10 days later and were classed as "potentially immune", and the remainder had no detectable antitoxin either before or 10 days after the injection of A.P.T. These horses were classified as "non-immune", but some might have possessed a low-grade potential immunity.

It is not possible to titrate at the Lr/100 level, under the conditions of this experiment, sera of lower value than about 0·2 unit per ml. The titres reached by the naturally immune horses, after a single injection, were sufficiently high for samples to be titrated for avidity. The potentially immunes and non-immunes, however, required two injections in order to produce a high enough titre for dilution-ratio measurements to be possible. Values of the dilution ratio for individual horses are given in table I: values for the immune horses are those after one injection, and for the other groups those after two injections of A.P.T.

It is clear that considerable differences in values were obtained in the different groups. Nearly all the naturally immune horses produced antitoxin of average or good avidity, six of the eight dilution ratios being over 1 0 All horses in the non-immune group produced non-avid antitoxin after two injections of A P T, the majority being very non-avid, all the dilution ratios for horses in this group were lower than any recorded in the naturally-immune group after one injection Only three potentially immune horses were injected, and the values in the second column of table I show that their average dilution ratio was intermediate between those for the other two groups

TABLE I

*Values of the dilution ratio of antitoxin produced by "normal" horses 10 14 days after injection with A P T*

| Naturally immune horses (after 1 injection) | Potentially immune horses (after 2 injections) | Non immune horses (after 2 injections) |
|---|---|---|
| 1 09 | 0 90 | 0 71 |
| 1 07 | 0 81 | 0 70 |
| 1 07 | 0 64 | 0 69 |
| 1 06 | | 0 65 |
| 1 04 | | 0 63 |
| 1 02 | | 0 60 |
| 0 94 | | 0 60 |
| 0 87 | | 0 50 |
| *Averages* 1 02 | 0 78 | 0 635 |

The results in table I strongly suggest that a relationship exists between the degree of immunity of the animals and the quality of antitoxin produced by them after injections It is perhaps surprising, however, that such a marked difference in dilution ratio should exist between non immune horses after receiving two injections of A P T at an interval of a month and other horses receiving one such injection after earlier stimulation from natural infection Several unknown factors are connected with naturally immune horses These include the number of natural stimulations and their duration together with the time between the last of these and the injection of A P T It is possible that relatively weak stimulation continued over a long period, as may occur in infection, may predispose an animal to produce antitoxin of good quality Further, the quality and nature of the antigen produced in infection in living tissue may differ considerably from that produced artificially in media

In order to determine whether the dilution ratio changed after the peak of production 10 14 days after injection, further blood samples were tested at various intervals, and the results are shown in table II Although a maximum error of about 10 per cent could theoretically be expected in the measurement of the dilution ratio, that obtained in practice is usually considerably lower. It was shown (Barr, 1949) in the determination of the dilution ratio of fourteen sera against five different toxins, that the ratio could be measured with extreme accuracy against one of the toxins The values obtained for the fourteen sera against this toxin all fell within ±3 per cent. of the expected values calculated from means involving all five toxins and all fourteen antitoxins. The toxin used in the present experiment was of the same general type as that referred to in 1949, and the greatest discrepancy from the mean value is under 5 per cent. (table II). It is therefore probable that none of these changes in dilution ratio was significant and that any change of 10 per cent. would be well outside the limits of experimental error.

TABLE II

*Values of the dilution ratio of diphtheria antitoxin in serum from horses bled at different times after injection with A.P.T.*

| Horse no. | Time after injection | | | Greatest discrepancy (per cent.) from mean value |
|---|---|---|---|---|
| | 10-14 days | 4 weeks | 6 or 8 weeks | |
| 7044 | 1·04 | 1·06 | 0·98 | 4·6 |
| 7039 | 1·02 | 1·06 | 1·01 | 2·9 |
| 7149 | 1·06 | 1·05 | 1·03 | 1·6 |
| 7145 | 1·07 | 1·08 | 1·06 | 0·93 |
| 7144 | 1·09 | 1·10 | 1·10 | 0·64 |
| 7000 | 0·90 | 0·89 | 0·83 | 4·9 |
| 7038 | 0·64 | ... | 0·67 | 2·3 |
| 7046 | 0·70 | ... | 0·68 | 1·4 |
| 7023 | 0·47 | ... | 0·46 | 1·1 |
| 7042 | 0·63 | ... | 0·62 | 0·80 |

### *The effect of successive injections on avidity*

Further injections of A.P.T. were given to some of the horses and the quality of the antitoxin again investigated by dilution-ratio determinations. The results obtained are shown in table III, which includes later values for a few other horses that responded too poorly for dilution-ratio measurements to be made after two injections. It will be seen that, within the limits of error of the test, there was no change in dilution ratio after the second injection of A.P.T. into the horses that had normal antitoxin at the start of the experiment. Subsequent injections brought about a slight steady increase in horse 7284; this horse had an extremely low normal titre before injection and might be expected to have had poor potential immunity at the start of the experiment.

It will be noted that four horses in the immune group received only two injections of A.P.T. Further injections were not given to these horses because they made considerable responses to the second injection; the stage reached after two injections was that of early hyperimmunisation, as compared with a stage of advanced prophylaxis reached after one injection. Whereas the average titre of these horses was 6·0 units per ml. after one injection, it rose to over 50 units after the second injection The two remaining horses received additional injections, and 7284 reached a value of 15 units only after the fourth and fifth injections , 7206, which had 40 units after the third injection, was intermediate between 7284 and the remainder It will be noted that the dilution ratio of sera from this horse remained remarkably constant after successive injections, and it appears from table III

TABLE III

*The effect of successive injections of A P T on the dilution ratio of antitoxin produced by horses*

| Horse no | Normal immunity state | Dilution ratio 14 days after injection no | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| | Naturally immune | | | | | | |
| 7144 | antitoxin 0 01 unit/ml | 1 09 | 1 02 | | | | |
| 7145 | „ 0 004 „ | 1 07 | 1 09 | | | | |
| 7149 | „ 0 04 , | 1 06 | 1 00 | | | | |
| 7044 | „ 0 002 „ | 1 04 | 0 96 | | | | |
| 7284 | „ 0 0005 „ | 0 94 | 0 93 | 1 00 | 1 08 | 1 11 | 1 12 |
| 7206 | „ 0 001 „ | | 1 03 | 1 02 | 1 02 | 1 01 | 1 00 |
| 7000** | Potentially immune | | 0 90 | 0 99 | 1 04 | 1 01 | 1 00 |
| 7038** | | | 0 64 | 0 90 | 1 06 | 1 03 | 0 98 |
| 7204 | | | 0 81 | 0 975 | 0 99 | 1 02 | 1 01 |
| 7203 | | | | 0 91 | 0 94 | 0 98 | 1 01 |
| 7207 | | | | 0 82 | 0 90 | 0 93 | |
| 7216 | | | | 0 75 | 0 825 | 1 01 | 1 00 |
| 7046** | Non immune | | 0 70 | 0 90 | 0 93 | 0 99 | 1 00 |
| 7042** | | | 0 63 | 0 99 | 1 01 | 1 07 | 0 94 |
| 7040** | | | 0 60 | 0 78 | 0 99 | 1 06 | 0 95 |
| 7023** | | | 0 47 | 0 89 | 0 99 | 1 01 | 1 06 |
| 7222 | | | 0 71 | 0 84 | 0 85 | 1 01 | |
| 7261 | | | 0 69 | 0 81 | 0 91 | 0 99 | 1 03 |
| 7288 | | | 0 65 | 0 79 | 0 94 | 1 00 | 0 94 |
| 7292 | | | 0 60 | 0 77 | 0 86 | 0 91 | 1 01 |
| 7285 | | | 0 50 | 0 68 | 0 66 | 0 87 | 0 91 |
| 7259 | | | * | 0 74 | 0 82 | 0 95 | 0 91 |
| 7283 | | | * | * | 0 58 | 0 76 | 0 79 |
| 7289 | | | * | * | * | 0 81 | 0 82 |

** Interval between second and third injections 4 months intervals for remainder, 1 month

* Antitoxic values too low for dilution ratio determinations

that, with the exception of 7284, the quality of antitoxin produced had reached a steady level after one injection Horse 7284 had the lowest normal titre and was the poorest responder in the group

The remaining horses, whether classified as potentially immune or non immune at the time of the first injection, produced antitoxin of better quality after successive injections, with a suggestion of a maximum level eventually being reached Although some of the increases in value of the dilution ratio may appear to be within the limits of error of the test, the progressively increasing trend gives significance to the changes recorded

There is a suggestion that a fall in dilution ratio may have occurred in some cases after reinjecting horses which had reached the stage of producing good quality antitoxin. This occurs to an appreciable extent in three of the naturally immune horses after their second injection and in a few of the original potentially immunes and non-immunes after the sixth injection. In the majority of such cases the *fall in dilution ratio is just within the estimated error of the test, but it* appears worth while to investigate the matter further. The dilution ratios recorded in table III are all those of samples taken two weeks after injections were given. It is possible that when potential immunity has reached a relatively high level, higher than that of the majority of horses whose values are shown in table II, changes in avidity may occur with time, in the absence of reinjection, and that the value of the dilution ratio of antitoxin two weeks after injection may be low. This tentative suggestion is based on results shown in the figure and discussed later, but it should be noted that in most cases in which a fall appeared to have occurred there was considerable antitoxin-production at this stage, as noted above, for some naturally immune horses after two injections.

The figures in table III suggest that initial differences in power to respond and in degree of potential immunity may play an important part in the determination of the number of injections necessary to produce antitoxin of good avidity. Evidence of considerable differences in potential immunity and power of so-called normal horses to respond to prophylactic injections of A.P.T. has already been furnished (Barr, 1950). It has already been pointed out, on the evidence provided in that paper, that some of the horses classified in tables I and III of this paper as non-immune may in fact have been potentially immune before any injections were given.

Consideration of table III shows that some of these " non-immune " horses (7046, 7042, 7040, 7023) produced antitoxin of slightly higher dilution ratio after the third and fourth injections than did some of the potentially immunes (7203, 7207, 7216). There are two possible reasons for this; the first that all these horses were actually potentially immune and that those classified as non-immune had the better power of response; alternatively, that the longer interval between the second and third injections given to the last-named horses favoured an earlier improvement in the quality of antitoxin produced. This difference in interval between injections may account for individual differences in the potentially immune group, and for the difference between the first four " non-immunes " and the remainder of the group.

It will be noted that dilution-ratio values are not recorded for some horses until they had received three, four or five injections. The reason for this was that titres produced earlier were too low for dilution-ratio measurements to be made. Such horses can therefore be regarded as relatively refractory to immunisation, and in general they required more injections than others in the same group before antitoxin of reasonably good avidity was produced. For example, the dilution ratio for horse 7289 (foot of table III) after five or six injections of A.P.T. was of the same order as that produced by many other horses after only three injections.

There is thus a strong indication that the number of injections necessary before antitoxin of good avidity is produced depends on the normal immunological state of the animal; this state includes the power of response to stimulation and the degree of potential immunity. It is possible that some animals are incapable of producing avid antitoxin.

### *Increase in dilution ratio with time*

It was concluded from the results shown in table II that no change in dilution ratio occurred after the peak of production 10-14 days after injection, over the period during which observations were made.

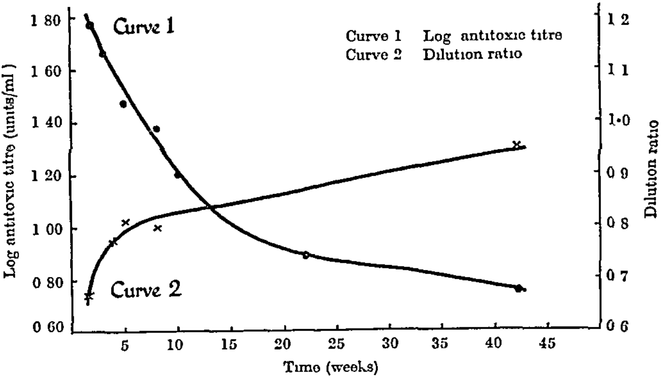

Fig.—The titre and dilution ratio of antitoxin in a naturally immune person at different times after a boosting dose of purified toxoid.

A definite increase was recorded, however, in blood samples taken from a person at different times after a boosting dose of purified toxoid. The antitoxic titres and values of the dilution ratio of these samples are shown in the figure: very non avid antitoxin was present two weeks after injection and a slow improvement occurred in course of time. There was insufficient serum to determine the dilution ratio of the 22 week sample.

It is possible that the first, rapidly produced antitoxin appearing after secondary stimulation of an imperfectly developed mechanism, may in certain circumstances be of the same loosely combining type as that produced in response to an earlier stimulation. Antitoxin of better avidity may conceivably be formed more slowly, after the initial hurried production has ended. Although no blood sample was taken before the 14th day after injection it is probable that very rapid production occurred in this person, because the value of the 14-day sample was 61 units per ml. and a considerable fall was recorded for some weeks before flattening of the curve occurred.

The absence of any measurable increase in the dilution ratio of antitoxin in the horses shown in table II may be due to certain conditions of the experiment, for example the use of a precipitated antigen. On the other hand, as suggested earlier in this paper, an increase in dilution ratio without further injection might occur in horses at a later stage in prophylaxis when basal immunity was better but still not fully established. It is interesting to note that the person in this experiment probably possessed good potential immunity; at the time of the injection he had 0·24 unit of diphtheria antitoxin per ml. though he had never received any injections of prophylactic previously.

## DISCUSSION

The results recorded here show that antitoxin produced in the very early stages of immunisation is of poor quality. This early antitoxin is only capable of fixing toxin loosely and not until a later stage can it neutralise firmly. This suggests that the production of non-avid antitoxin in hyperimmunisation may frequently be due to the forcing of the antibody-producing mechanism before it is fully equipped. A horse insufficiently prepared for hyperimmunisation may be unable, under the strain of frequent, increasing doses of antigen, to increase the output of antitoxin and at the same time improve its quality. Hyperimmunisation may thus result in the production of antitoxin of low titre and poor avidity, for it has been shown (Barr and Glenny, 1945-46) that there is a relation between quantity and quality in the hyperimmunisation of horses producing diphtheria and tetanus antitoxins. A relation was found between the antitoxic titre and the serum ratio.

It was suggested (Barr, 1950) that the term " basal immunity " might well be reserved for a standard of immunological preparation of an animal adequate for a particular purpose. This should at all times include a high degree of potential immunity, but it now appears that for hyperimmunisation an adequate standard is reached only when the animal is producing antitoxin of good quality.

It is well known that in the production of tetanus antitoxin in non-immune horses rest is necessary after the preliminary injections given to establish basal immunity. If hyperimmunisation is started before this basal immunity is well established, antitoxin of low titre and poor avidity results. During the period of rest it is probable that the antitoxin-producing mechanism continues to develop in the process of continued production without injection, and that the quality of the antitoxin formed slowly improves with time. That this phenomenon can occur very soon after secondary stimulation is shown in the figure. The effect of a course of injections given before this improvement had been achieved might be a delay in reaching this state through the forcing of an imperfectly operating mechanism, insufficiently developed to deal with large quantities of antigen. It is shown in table III that a delay of 3 months from the second to the third injection of A.P.T. may be beneficial regarding the quality of antitoxin produced.

Petrie attempted to prepare tetanus antitoxin in a horse that had not received the usual preparatory doses of toxoid followed by a resting period. Although an efficient antigen was used, the antitoxic titre reached was under 50 International units (1928) after two courses of immunisation. It is clear that the antitoxin was extremely non-avid, for the ratio of the value found by testing in mice to that found in guinea-pigs was $>340/100$, " the most discrepant inverse ratio met with " in a piece of work dealing with the antitoxic values of sera as measured by these methods using a number of different toxins. It is unfortunate that in this valuable work the test doses of toxin were fixed against a non-avid antitoxin (a former British standard). Because of this, the majority of sera used, which were of average avidity, gave lower titres by the mouse test than by the guinea-pig test, and no satisfactory explanation was forthcoming. The reason for this finding must have been the use of too low a test dose in the guinea-pig titrations in relation to that used in the mouse titrations. These doses had been fixed against an abnormal antitoxin which combined loosely with toxin, so that considerable dissociation occurred after injection into the larger animal, in consequence of which an abnormally low guinea-pig test dose was adopted. With one of the toxins, the ratio of the test doses (guinea-pig/mouse) was 0·70 ; of nineteen sera tested,the range of ratios of apparent antitoxic values (mouse/guinea-pig) was 0·58 to 1·51, and twelve were between 0·63 and 0·78, nine of these within 5 per cent. of 0·69. If one of these antitoxins had been used to fix the test doses, the majority would thus have had a ratio of approximately 1·0, and, had the ratio been inverted (guinea-pig value/mouse value), the values obtained for non-avid antitoxins would have been less than 1·0, which seems more reasonable for any ratio used to measure avidity.

Evidence of change in quality of other types of antibody during immunisation has been provided by various workers. Thus Pappenheimer (1940) obtained a non-precipitating antibody in the early stages of immunisation of a horse with egg albumen. Heidelberger and Kendall (1935) found that a fraction of low-grade or incomplete antibody was present in all rabbit anti-egg albumen sera ; this material was incapable by itself of producing precipitates with antigen. This finding may be compared with the earlier conclusions of Barr and

Glenny (1931) and Glenny and Barr (1932*a*) in the fractionation of diphtheria antitoxic serum from hyperimmunised horses. Fractions of different quality were prepared from a single serum, suggesting that all sera might contain antitoxin of different grades of avidity. Kekwick and Record (1941) found that the diphtheria antitoxin associated with the $\gamma$-globulin fraction of a hyperimmunised horse differed in certain properties from that in the $\beta$-globulin fraction: the serum ratios differed, and the composition of the floccules formed with toxin. They suggested that the relative proportions of the $\beta$ and $\gamma$ components might influence the qualitative properties of different sera. With the exception of Pappenheimer's work, these observations were made on sera from well-immunised animals.

It is not known whether qualitative differences observed by in-vitro and by in-vivo methods have the same fundamental significance. At the present time, avidity of antitoxins in the in-vivo sense cannot be estimated by an in-vitro test alone; moreover it is well known that precipitation or flocculation is affected by chemical and physical conditions. Hartley (1925) showed that after ether extraction a diphtheria antitoxic serum would no longer flocculate with toxin, though its power of neutralisation remained.

It seems possible, however, that the first antibody appearing in the circulation may be "immature" as suggested by Petrie; this immaturity might consist in a deficiency in specific combining groups. As immunisation proceeds, or as the antibody-producing mechanism becomes more efficient, the number or spacing of the combining groups may approach that required for the ideal firm neutralisation of toxin by the antitoxin molecule. The first-produced antitoxin might thus be an ill-formed antibody to a well-defined antigen.

On the other hand, the heterogeneous nature of toxins and toxoids, as well as of antitoxins, requires serious consideration. Qualitative differences exist among crude toxins and toxoids, both as regards antigenic efficiency and, among toxoids, binding power for antitoxin. It has been shown that the affinity of toxoids for antitoxin is roughly correlated with their antigenic efficiency both in guinea-pigs and in the hyperimmunisation of horses, and that the affinity of toxoids for non-avid antitoxin is low (Barr and Glenny, 1949). Differences in "goodness of fit" have been observed between certain samples of diphtheria toxin and antitoxin (Barr, 1949) and tetanus toxin and antitoxin (Petrie; Glenny and Stevens, unpublished). This may be shown by the finding of abnormally high or low values of certain antitoxins when titrated against certain toxins, and Petrie obtained indications of higher antitoxic values when sera were titrated against the same toxin or type of toxin as that used in the immunisation of the animal.

All these findings can be regarded as evidence of variation or heterogeneity among antigens, and an example of abnormal behaviour *in vitro* was recorded by Barr and Glenny (1938). A certain diphtheria antitoxin of serum ratio 3 14 gave a constant titre when tested by flocculation against eight different batches of toxin When tested against twenty-three different toxoids, however, higher values were invariably obtained, the serum appearing from 9 to over 40 per cent stronger than the accepted constant value recorded against unmodified toxins

It seems clear that the affinity of different antitoxins for toxoids shows variation All culture filtrates contain natural toxoid, and it is possible that the presence of this material, which may vary in quality as well as in quantity, may be responsible for differences in goodness of fit or firmness of combination

Petrie suggested that crude tetanus toxins could be regarded as mixtures of a primary toxin molecule and an antigenic variant of it Tetanus antitoxic serum would likewise consist of mixtures, in varying proportions, of the corresponding antitoxins Barr and Glenny (1949) suggested that different molecules of an antigen might have a common basic structure with slight steric differences which assisted or hindered firm combination with antitoxin A similar state of affairs might exist among antitoxin molecules

It is thus possible that loose combination might occur between various slightly dissimilar antigen and antibody molecules and that at all stages of immunisation antitoxic sera consist of a mixture of well formed antibodies to a number of slightly different antigens As immunisation proceeds there would presumably be an increase in the proportion of the type of antibody which combines most firmly with the main toxic constituent of the majority of test toxins

## SUMMARY

Diphtheria antitoxin produced after two injections of A P T into non immune horses or horses with feeble potential immunity is non avid The avidity progressively improves as further injections of antigen are given and in many instances reaches a steady level

Although changes in avidity were not observed in these horses if they were not reinjected, a definite progressive improvement with time was observed in serum from a person after a single boosting dose This may be due to very rapid production in a person with good potential immunity

The number of injections necessary to produce antitoxin of good avidity depends on the normal immunological state of the horse, *i e* capacity to respond, and degree of potential immunity The spacing of injections may also play a part

It is probable that the production of non avid antitoxins in hyperimmunisation is frequently due to the forcing of an incompletely developed antitoxin producing mechanism

The early non avid antitoxin may be an incomplete or ill formed antibody to a single antigen, or a heterogeneous collection of well-formed antibodies to a number of slightly dissimilar antigen molecules, As immunisation proceeds the antibody may become more complete, or if non-uniform, changes might occur in the proportions of different antibodies relative to the proportions of corresponding antigens in toxins.

The non-avid human antitoxin was found in the course of a long-term investigation with Dr A. C. Cunliffe of King's College Hospital. I am indebted to Mr A. Thomson for the injection of the horses and to Mr A. T. Glenny, F.R.S., who has spent some time discussing these results with me. I also wish to thank Miss F. Anderson, Mrs B. Cripps, Miss D. Delahoy and Miss F. Webb for technical assistance.

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576 . 322 : 576 . 852 . 27 (*Lœfflerella pseudomallei*)

# FLAGELLATION AND TAXONOMY OF WHITMORE'S BACILLUS

C. S. BRINDLE and S. T. COWAN

*National Collection of Type Cultures, Colindale Avenue, London*

(PLATE XCIV)

POLAR flagellation is characteristic of bacterial genera in the family Pseudomonadaceæ, and peritrichous flagellation of motile species in the families Enterobacteriaceæ and Parvobacteriaceæ. Whitmore's bacillus is classified in the Parvobacteriaceæ by Breed *et al.* (1948) and in the Mycobacteriaceæ by Wilson and Miles (1946) but in the descriptions of the organism the type of flagellation is not mentioned. We decided to stain the flagella of strains in our Collection; the technical difficulty of doing this probably accounts for the lack of information in the literature.

## CULTURES AND METHODS

*Strains.* We have eight strains of Whitmore's bacillus; their source and date of isolation are shown in the table Culturally, all the strains are typical but the older ones have lost much of their ability to ferment carbohydrates; all are agglutinated by serum from rabbits immunised against strain 7431. We have not made any animal experiments to determine pathogenicity, but all the strains were known to be pathogenic to guinea-pigs before deposition

TABLE

*Source of strains*

| Strain | | Date isolated or received | Source | Reference |
|---|---|---|---|---|
| N C T C no | Name | | | |
| 1688 | Rat | 1923 (R) | Melioidosis in rat | Stanton and Fletcher (1924-25) |
| 1691 | Ragaviah | 1923 (R) | Melioidosis in man | „ |
| 4845 | S 397 | 1935 (I) | Monkey (Singapore) | . |
| 4846 | S 396 | 1935 (I) | Monkey (Singapore) | .. |
| 6700 | Haigh 11787 | 1942 (I) | Abscess (England) | Grant and Barwell (1943) |
| 7383 | Harries | 1948 (R) | Melioidosis in man (*Burma*) | Harries *et al.* (1948) |
| 7431 | Guy's | 1948 (I) | ... | Jameson (1949) |
| 8016 | AHS 1 | 1949 (R) | Sheep in Australia | ... |

(I) = date of isolation

(R) = date received in N C.T.C. if date of isolation is not known in the National Collection of Type Cultures. To increase motility, Craigie tubes in semi-solid glycerol agar were inoculated (Craigie, 1931) and incubated at 30° C.

*Preparation of films.* Cultures were grown on nutrient agar slopes for 10-15 hours at 30° C., and films prepared by a method shown to us by Mr E. Clark of Queen Charlotte's Hospital. A drop of distilled water was put in the centre of a grease-free slide; the growth was touched with a straight wire and the tip put in the drop of water. After the wire tip had been in the water for 1-2 minutes the holder was lowered so that it was horizontal and the distal end of the wire was in contact with the slide. The drop spread along the wire, and by movements to the right and to the left it was spread as a thin film, which was allowed to dry in the air.

An alternative method was used for strains that were not actively motile on nutrient agar. These were grown for 15-18 hours at 30° C. in peptone water containing 0·5 per cent. glycerol. The culture was centrifuged lightly and four-fifths of the supernatant fluid removed. Sterile distilled water was added to replace about half of the fluid withdrawn. Incubation was continued for 1 hour at 30° C. and a loopful spread by Clark's method.

*Fixing and staining.* Films were fixed in Kirkpatrick's mercuric fixative (Muir and Ritchie, 1927) for 1-2 minutes, and then washed with water. Kirkpatrick's mordant (Browning and Mackie, 1937, p. 124) was applied for 15-20 minutes, the film washed, and the slide dried on edge. Kirkpatrick's silver solution (Browning and Mackie, p. 125) was filtered on to the dry film, heated gently until a metallic scum appeared on the edge of the film (usually 10-15 seconds), and the stain allowed to act for another 30-60 seconds, after which it was flooded off with water. Finally, the film was stained for 5 minutes with carbol-fuchsin diluted 1 : 10, washed, dried, and mounted in neutral Canada balsam or DePeX mounting fluid.

## RESULTS

We examined hundreds of films and usually found that although the flagella were stained they were detached from the bacillary bodies and formed tangled masses. We concentrated on young, actively motile cultures and on methods of making films that were least likely to detach the flagella. The methods described gave the best results, but at least ten films had to be made and examined to find one rod with attached flagella.

Eventually we stained flagella on seven of our strains, and the flagellar attachment was always polar. Usually there were two flagella at one pole but sometimes they were more numerous; occasionally flagella were seen at both poles. The flagella were so fine that they did not present good contrast for photography unless they were twisted together near the point of origin (figs. 1 and 2).

## TAXONOMY

We believe our cultures were authentic strains of Whitmore's bacillus; if that is accepted, we have established that the flagellation of this species is polar. The taxonomic position of the species must be reconsidered in the light of our findings.

Stanton and Fletcher (1924 25) produced good evidence that Whitmore's bacillus was closely related to the glanders bacillus and most taxonomists now include both organisms in the same genus Pons (1927) suggested that it occupied a position intermediate between the glanders bacillus and *Pseudomonas pyocyanea* , Legroux and Genevray (1933) thought that Whitmore's bacillus was peritrichously flagellated, and decided that, apart from the absence of a serological relationship, the species was more closely allied to *Ps pyocyanea* than to the glanders bacillus The similarity between Whitmore's bacillus and the blue pus organism seems to have confused Castellani who, in describing a new species before the Societe Portugaise de Biologie on 8th July 1948, said " Dans ma opinion, le microorganisme est très pres du *Malleomyces pseudomallei* (Bacille de Whitmore), et il peut être placé le même genre (Malleomyces) " (manuscript kindly supplied by Dr A Castellani) We examined a culture of Castellani's new species, *Malleomyces hæmorrulcogenes*, but we thought it was a strain of *Ps aeruginosa* (*Ps pyocyanea*) Dr W C Haynes (personal communication) of the Northern Regional Research Laboratories, Peoria, Illinois, agrees with our determination

Our finding that the flagellation of Whitmore's bacillus is polar strengthens the argument for a close relationship to the genus Pseudomonas, and virtually forces the species into the family Pseudomonadaceæ , it can either be incorporated in a genus already in that family, or the family can be enlarged to include the genus of which it is a species Only two genera of the Pseudomonadaceæ need to be considered Pseudomonas and Xanthomonas Pseudomonas species are usually characterised by the production of water soluble pigments but non pigmented species occur as parasites in the animal and plant worlds It would be wise to exclude Whitmore's bacillus on the ground that although it is a pigment producer the pigment is not soluble in water Xanthomonas is an off shoot of Pseudomonas and was created by Dowson (1939) for pseudomonads that do not show the fluorescent colonies of Pseudomonas species, but produce a yellow pigment insoluble in water Whitmore's bacillus often shows a yellow pigmentation of its colony and has many other characters in common with Xanthomonas species, but until thorough comparisons have been made between Whitmore's bacillus and these plant pathogens we do not think it prudent to include Whitmore's bacillus in this genus

We are left without a choice , we must enlarge the family Pseudomonadaceæ to include the genus of which Whitmore's bacillus is a species We agree with Stanton and Fletcher that Whitmore's bacillus is closely related to the glanders bacillus , the relationship is that of two species in the same genus It follows, therefore, that if one species is translated to the family Pseudomonadaceæ, the whole genus must go with it

Stanton and Fletcher (1924-25) produced good evidence that Whitmore's bacillus was closely related to the glanders bacillus, and most taxonomists now include both organisms in the same genus. Pons (1927) suggested that it occupied a position intermediate between the glanders bacillus and *Pseudomonas pyocyanea*; Legroux and Genevray (1933) thought that Whitmore's bacillus was peritrichously flagellated, and decided that, apart from the absence of a serological relationship, the species was more closely allied to *Ps. pyocyanea* than to the glanders bacillus. The similarity between Whitmore's bacillus and the blue-pus organism seems to have confused Castellani who, in describing a new species before the Société Portugaise de Biologie on 8th July 1948, said " Dans ma opinion, le microorganisme est très près du *Malleomyces pseudomallei* (Bacille de Whitmore), et il peut être placé le même genre (Malleomyces) " (manuscript kindly supplied by Dr A. Castellani). We examined a culture of Castellani's new species, *Malleomyces hæmorrulcogenes*, but we thought it was a strain of *Ps. aeruginosa* (*Ps. pyocyanea*). Dr W. C. Haynes (personal communication) of the Northern Regional Research Laboratories, Peoria, Illinois, agrees with our determination.

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## NOMENCLATURE

The classification of a bacterium is a matter of opinion; nomenclature is much simpler. Matters of opinion hardly arise as we are guided by the principles, rules, and recommendations of the bacteriological code (Buchanan, St John-Brooks, and Breed, 1948).

Whitmore (1913-14) named his organism *Bacillus pseudomallei*; Stanton and Fletcher (1921), without producing evidence, changed this to *Bacterium whitmori* on the ground that the specific epithet " pseudomallei " was invalid. The genus Bacillus, circumscribed by international agreement (Second Internat. Congr. Microbiology, 1936), excludes Whitmore's bacillus which, on transfer to another genus, must have its original specific epithet re-established (Rule 18). This epithet, " pseudomallei," " must not be rejected, changed, or modified merely because it is badly chosen or disagreeable, or because another is preferable or better known " (Rule 23).

The generic name is that of the type species of the genus, in this case the glanders bacillus. The glanders bacillus has been included in many different genera (see Breed *et al.*, 1948) but Pfeifferella (Buchanan, 1916, 1918) was the first genus of which it was the type species. The name Pfeifferella was a clerical error (Buchanan, 1925) and is an illegitimate later homonym of Pfeifferella Labbé (Becker, 1951). Bergey *et al.* (1939) suggested Malleomyces Pribram but as this is a later homonym of Malleomyces Hallier, it seems to us that Lœfflerella (Gay *et al.*, 1935) is the valid name of the genus, and that the complete citation should be *Lœfflerella pseudomallei* (Whitmore) Gay *et al.*

## SUMMARY AND CONCLUSIONS

1. Flagella were stained on seven of eight strains of Whitmore's bacillus. In every strain the attachment of the flagella was polar.

2. The species is closely related to the glanders bacillus (a non-motile organism) and the type of flagellation warrants inclusion in the family Pseudomonadaceæ.

3. The two species should form a genus, but at present this genus should not be combined with any accepted genus of the family Pseudomonadaceæ.

4. The complete citation of Whitmore's bacillus is *Lœfflerella pseudomallei* (Whitmore) Gay *et al.*

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TAXONOMY OF WHITMORE'S BACILLUS

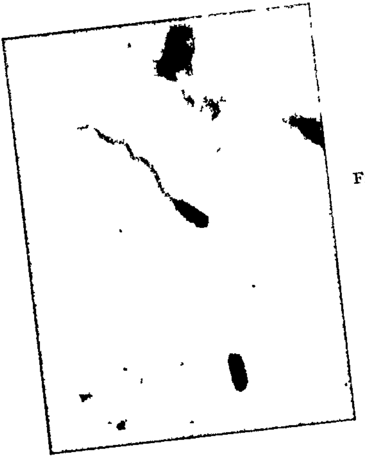

FIG. 1.—Strain 8016, showing deeply stained tuft of polar flagella. Kirkpatrick's silver stain. ×3000.

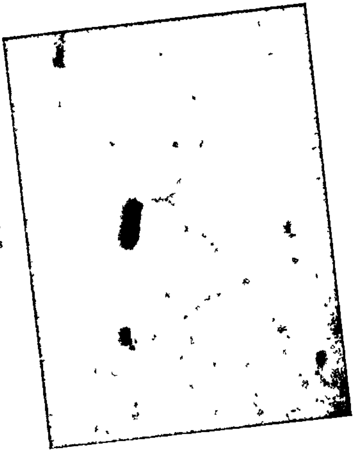

FIG. 2.—Strain 7431, showing flagella twisted together at proximal end. Kirkpatrick's silver stain. ×3000.

| | | |
|---|---|---|
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616 . 126—002—02 · 576 . 852 23

# CORYNEBACTERIAL ENDOCARDITIS: WITH REPORT OF A CASE DUE TO TOXIGENIC *CORYNEBACTERIUM DIPHTHERIÆ*

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The isolation of *C. diphtheriæ* from the blood stream of cases of clinical diphtheria during life has been reported by a number of workers since von Niessen (1902) made the first observation.

Roedelius (1913) isolated the organism in three out of 187 cases investigated. Elkeles (1929) reported two cases of diphtheria with positive blood cultures and in one the organism was shown to be toxigenic. A gravis type *C. diphtheriæ* was grown from the blood 26 hours before death in a case of faucial diphtheria described by Chi Wu and Ts'un (1936).

In some cases, however, the blood cultures gave mixed growths. Thus Leede (1911) isolated *C. diphtheriæ* together with an hæmolytic streptococcus from one of 18 cases of diphtheria, and one of three cases described by Roedelius gave a streptococcus in addition to *C. diphtheriæ*.

Some authors give only scanty bacteriological data (Ucke, 1908; Leede, 1911; Morgan, 1913) so that proof that the organism isolated was in fact *C. diphtheriæ* is lacking. Indeed, Zinsser and Bayne Jones (1934) considered that there was no evidence that true *C. diphtheriæ* septicæmia could occur in life.

There is an extensive literature, largely German, on the isolation of *C. diphtheriæ* from the blood and organs at autopsy in cases of diphtheria. Loeffler (1884) reported the isolation of *C. diphtheriæ* from the liver at autopsy in a number of cases. Leede (1911, 1913) found *C. diphtheriæ* in heart blood *post mortem* in four cases out of 62, and in eight cases out of 130 that had had tracheotomy. Mixed cultures also occurred, and evidence for the identity of the organisms reported as *C. diphtheriæ* is often insufficient. Elkeles isolated *C. diphtheriæ* from the organs at post mortem in 33 of 64 cases of diphtheria. He regarded the occurrence of the organisms in the internal organs as an agonal phenomenon.

A patient with faucial diphtheria who developed endocarditis was observed by Chiari (1933). Blood cultures were not made during life, but at autopsy the presence of endocarditis was confirmed, and *C. diphtheriæ*, virulent for guinea pigs, was isolated from the vegetations on the tricuspid valve. He reported a similar case two years later (Chiari, 1935). Virulent *C. diphtheriæ* was isolated by Lenti and Wirz (1938) from the blood of a patient with faucial diphtheria on the day before death. At autopsy, mitral endocarditis was found and organisms were demonstrated in sections of the vegetations.

Kessel and Romanoff (1930) described the isolation of *C. hoffmani* from both the blood and the cerebrospinal fluid in a case of meningitis. No mention of endocarditis was made, and this patient recovered. In a series of 730 patients submitted to diagnostic blood culture, Thompson (1932) found diphtheroids

616 . 126—002—02 : 576 . 852 . 23

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Kessel and Romanoff (1930) described the isolation of *C hoffmanni* from both the blood and the cerebrospinal fluid in a case of meningitis No mention of endocarditis was made, and this patient recovered In a series of 730 patients submitted to diagnostic blood culture, Thompson (1932) found diphtheroids in 30. One of these had endocarditis both clinically and at autopsy, but although the primary culture grew small bacilli, subcultures produced typical streptococci. Pleomorphic streptococci that can resemble diphtheroids are well known, and were used by Welch *et al.* (1935-36) to produce experimental endocarditis in rabbits.

That diphtheroids from the skin may appear in blood cultures as contaminants when technique is faulty is of course universally recognised. Tow and Wechsler (1932) reported a case of endocarditis, confirmed at autopsy, in which *C. hoffmani* was isolated from the blood during life. Cocci, however, were also recognisable in sections, and in the final paragraph the authors conclude that the cocci were the causative organisms. Babes and Manolesco (1908) found an organism resembling *C. diphtheriœ* together with a streptococcus in the aortic vegetations of a case of endocarditis.

Finally, a number of cases of endocarditis due to *C. diphtheriœ* have been reported in which the patient did *not* manifest clinical diphtheria. In most of these, however, the organism was not toxigenic. Howard (1893) observed a man of 44 with diarrhœa and pyrexia who died from circulatory collapse. At autopsy, endocarditis was found, and an organism indistinguishable from *C. diphtheriœ* was isolated which proved to be avirulent for guinea-pigs and rabbits. Roosen-Runge (1903) reported a case of mitral endocarditis due to a non-toxigenic *C. diphtheriœ* and another of tricuspid endocarditis due to a similar organism (Roosen-Runge, 1925). Sutherland and Willis (1936) described a case of endocarditis with isolation of a non-toxigenic *C. diphtheriœ* both from the blood during life, and from the vegetations and organs at autopsy. Kaschel (1937-38) reported a similar case with mitral vegetations and isolated a non-toxigenic *C. diphtheriœ.*

Very few cases are on record of bacterial endocarditis due to a toxigenic *C. diphtheriœ* in patients showing no clinical evidence of diphtheria. Herzog (1918) described a patient with pyrexia in whom mitral vegetations were found at autopsy. On culture they yielded a toxigenic *C. diphtheriœ,* but no cultures were made during life. Buddingh and Anderson (1937) reported a case of recurrent epistaxis in a boy of 16 years. At autopsy mitral endocarditis was found and cultures grew a toxigenic *C. diphtheriœ.* A Gram-positive bacillus had been grown from the blood during life, but was not further identified.

The following case falls into this last category, and appears to be the third in the literature.

## CLINICAL HISTORY AND COURSE

A Canadian infantryman aged 24, serving in the Italian theatre, had been fit and well until 16th January 1944, when he developed abdominal pain and anorexia. On the 19th he was admitted to a medical unit where his temperature was found to be 100° F. A blood slide examination showed infection with *Plasmodium falciparum* and a course of quinine, gr. 10 *t.d.s.*, was started. By the 23rd the temperature appeared to have settled and mepacrine was substituted for quinine. On the 24th the temperature rose to 103·4° F. and *P. falciparum* was again found in a blood film. Quinine was given intravenously and repeated on the following day. Sulphathiazole was also started on the 24th as adventitous sounds were heard in the right chest. By the 29th the temperature had settled; sulphathiazole was stopped but mepacrine was continued.

since admission. On the 13th, the corynebacterium from the first blood culture was shown to be morphologically and culturally indistinguishable from *C. diphtheriæ.* Accordingly 80,000 units of antidiphtheritic serum were given intravenously. On the 14th, the drip was discontinued and sulphapyridine given by mouth, 6 g. *per diem.* An organism had now been isolated from the second blood culture indistinguishable from the first.

During the 15th the general improvement was maintained. There was now no pain or tenderness in the chest or abdomen. The apical murmur became more marked. Ophthalmoscopy showed a circular hæmorrhage with a white centre near the upper temporal branch of the left retinal artery. During the evening of the 16th the temperature rose again to 103° F., but there was no change in the general condition. Blood slides were negative for malarial parasites. The temperature remained at about 100° F. until the 20th, when the sulphapyridine was discontinued after a total of 60 g.

On the 21st, at 09.00 hrs., the patient was restless and complained of numbness in the right leg, and his pulse rose to 120 and the temperature to 102·5° F. The right femoral pulse could not be felt. The left was easily palpable. The right femoral artery appeared to be in spasm and could be felt as a cord. The popliteal and dorsalis pedis pulses could not be felt on either side. All forms of sensation were diminished below the right knee. The plantar reflexes were flexor. By 15.00 hrs. there was distinct cooling of the right foot and pallor of the toes. On the following day the blood pressure in the left thigh was 140/100 mm.Hg., but not recordable on the right. A third blood culture was taken.

The temperature remained around 102° F. until the 26th. During this time the blood was negative for plasmodia, the urine contained albumin, urobilin, scanty leucocytes, red cells and granular casts. Examination of a loose stool revealed no blood, mucus or parasites. A corynebacterium had been isolated from the third blood culture. On the 26th, examination showed no difference in the temperatures in the feet, improved movements on the right side, and numbness confined to the distal half of the right foot. The right femoral pulse was just palpable.

During the evening of the 26th the patient collapsed, but improved after an injection of Nikethamide. He was noticed by the night sister to be aphasic and hemiplegic. Examination on the 27th showed complete right hemiplegia and dysarthria. There was lateral deviation of the left eye and the general condition was very poor. On the 28th the patient lapsed into a stuporose condition which persisted until his death. On the 1st March the temperature rose terminally to 105° F. and he died at 03.40 hrs., six weeks after the onset of illness.

## BACTERIOLOGY

The organism isolated from the blood possessed the following characteristics :—

1. Profuse growth in glucose broth and, surprisingly, in bile-salt water.
2. Morphology : Gram-positive bacilli arranged in Chinese figuring showing metachromatic staining with methylene blue followed by acetic acid ; morphologically indistinguishable from *C. diphtheriæ.*
3. Colonies on horse blood-agar after 24 hours were pale, smooth, regular, about 0·1 cm. in diameter, surrounded by a narrow zone of hæmolysis.
4. Colonies on Hoyle's tellurite medium at 48 hrs. were smooth, round, dark grey in colour, and about 0·1 cm. in diameter.
5. Broth cultures showed pellicle formation with a granular deposit.
6. Biochemical reaction (serum-water sugars) :—

| Carbohydrate | Acid | Clot |
|---|---|---|
| Glucose . . . . . . | + | + |
| Saccharose . . . . . | — | — |
| Starch . . . . . . | + | + |
| Lactose . . . . . . | — | — |

7. On blood-agar the organism was inhibited by sodium sulphapyridine using the gutter plate method.
8. Virulence tests (guinea-pigs) :
   - (*a*) Intradermal tests : Eight were performed using three separate test animals and three separate control animals. In all tests the organism appeared to be virulent.
   - (*b*) Injection of whole cultures : Two test animals died ; two control animals protected with 1000 units of diphtheria antitoxin intraperitoneally survived. Autopsy on animals dying showed pleural effusions, hæmorrhages in the suprarenals, and inflammatory œdema at the inoculation sites.

The organism was therefore considered to be a virulent, toxigenic gravis strain of *Corynebacterium diphtheriæ.*

## AUTOPSY FINDINGS

Autopsy was performed six hours after death and showed :—

*External appearances.* A few bluish macules were recognisable over the chest and abdomen, and two bluish patches over the right great toe. The right leg was rotated outwards. A small conjunctival since admission. On the 13th, the corynebacterium from the first blood culture was shown to be morphologically and culturally indistinguishable from *C. diphtheriæ*. Accordingly 80,000 units of antidiphtheritic serum were given intravenously. On the 14th, the drip was discontinued and sulphapyridine given by mouth, 6 g. *per diem*. An organism had now been isolated from the second blood culture indistinguishable from the first.

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infarcts. The rest of the spleen was pinker than normal and the pattern was indistinct. The tracheal lymph nodes were deeply pigmented. The other nodes, the thymus, thyroid, pituitary and suprarenals appeared normal.

*Genito-urinary system.* Both kidneys contained many yellow cortical infarcts from 1·5×1·0 cm. to 0·5×0·5 cm. The ureters, bladder, prostate and testicles were normal.

*Central nervous system.* No macroscopic changes could be seen in any part of the nervous system, but the tip of the left temporal pole and the cortex of the left insula were softer than those on the right side.

## HISTOLOGY

*Lateral wall of the left ventricle.* Interstitial myocarditis, with foci of neutrophils, eosinophils and lymphocytes between muscle fibres. Mild subacute inflammation of pericardial fat.

*Thinned apex of the left ventricle.* In some areas myocardial fibres have disappeared and have been replaced by fine connective tissue with some lymphocytes and scanty neutrophils. Many dead and degenerate myocardial fibres are present.

*Mitral valve.* A pedunculated vegetation consisting of a granular stroma containing masses of organisms, erythrocytes and leucocytes. The organisms were arranged in colony-like clumps, which in the central zone appeared to be dead. At the edge of the valve there was fibroblastic proliferation and neutrophil infiltration. Numerous corynebacteria indistinguishable from *C. diphtheriæ* were demonstrated by the Claudius method.

*Occluded branch of coronary artery.* Destruction of intima and media through most of the circumference, with rupture of the internal elastic lamina. The adventitia and the remains of the media were infiltrated with neutrophils. The vessel contained a thrombus with numerous organisms. The adjacent pericardial fat was inflamed. Typical corynebacteria were demonstrated in thrombus by Claudius's method.

*Femoral artery embolus.* Granular stroma with numerous included clumps of organisms and some surface neutrophils.

*Lung.* Congestion and œdema with typical bronchopneumonic lesions.

*Liver.* Central congestion and atrophy. Bile pigment in central cells. Slight fatty degeneration irregularly scattered.

*Spleen.* The spleen was congested and contained numerous areas of necrosis. In some of these areas there were large masses of bacilli. Granulation tissue was present at the edges of some necrotic zones. Large numbers of neutrophils were present in the pulp. Malpighian bodies were reduced in size. Thromboses were present in many venous sinuses and organising fibrin was present on the capsular surface.

*Tracheal lymph node.* Congestion and œdema with sinus catarrh. Moderate amount of carbon pigment present.

*Thyroid.* Normal.

*Suprarenal.* Areas of degeneration in the zona fasciculata.

*Kidney.* The kidney contained a pale, triangular infarct of the cortex with a surrounding zone of hyperæmia. An artery at the apex of the infarct contained organising thrombus with early recanalisation. Elsewhere most of the glomeruli appeared normal, but a few showed adhesive glomerulitis. The convoluted tubules showed parenchymatous degeneration.

*Cerebral cortex.* Areas of œdema were present with swelling of oligodendrocytes.

## Discussion

In the present case the diagnosis was at first obscured by the pre-admission reports of the finding of *Plasmodium falciparum.* Thereafter the case progressed as one of bacterial endocarditis with repeated embolic phenomena.

It is unfortunate that pharyngeal and nasal cultures were not made. At no time, however, did the patient have symptoms referable to the nose or throat. In this respect the case resembled that of Herzog. Repeated clinical examinations were made in life and were always negative. Neither were any lesions found in the nose or pharynx at autopsy. There were no cutaneous wounds or sores. The portal of entry must therefore remain problematical.

There was no evidence of previous rheumatism, nor of congenital abnormalities of the cardiac valves.

The administration of serum seemed to have little effect on the course of the disease. The only drug that appeared to affect the pyrexia was sulphapyridine, and in-vitro tests showed that the organism was inhibited by the drug (penicillin was not at the time available).

The identification of a toxigenic *Corynebacterium diphtheriæ* as the cause of the endocarditis is regarded as established. The three blood cultures all gave pure growths of the organism. Neither were any other organisms seen in any of the numerous histological preparations showing *C. diphtheriæ.*

It seems clear that corynebacteria isolated from blood cultures should not be lightly dismissed as contaminants.

## Summary

The occurrence and distribution of corynebacteria within the body before and after death are discussed.

A case of bacterial endocarditis due to a toxigenic *C. diphtheriæ* in a patient showing no evidence of clinical diphtheria is described, and an account is given of the bacteriological and post-mortem findings.

I wish to express my thanks to Dr A. C. Dornhurst (formerly Medical Specialist, R.A.M.C.), under whose care the patient was, for the clinical notes of the case

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616 . 133 . 27—002 . 2—018 . 215

# A FATAL CASE OF GIANT-CELL OR TEMPORAL ARTERITIS

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(Plates XCV-XCVII)

The literature of giant cell or temporal arteritis has recently been reviewed by Harrison (1948) and Migone and Mortara (1949) In addition to the 122 cases collected by the latter authors 14 other cases have been reported Hutchinson (1889 90), Vandivier and Ritchey (1945), Anderson *et al* (1948), Moss (1948), Wolnetz *et al* (1948), Zeller (1948), Hauss and Burwinkel (1949), Keen (1950) and McMillan (1950) In view of the paucity of necropsy observations it is considered worth while to record a further case diagnosed clinically as temporal arteritis on which a full post mortem examination was performed

## Nomenclature

Horton *et al* (1932) introduced the term "temporal arteritis" in the belief that the disease was localised in these vessels Kilbourne and Wolff (1946), in view of the clinical involvement of cerebral and retinal vessels, suggested the term "cranial arteritis" The histological findings have resulted in the introduction of further names — "giant-cell arteritis" by Gilmour (1941), and in relation to the aortic lesions, "chronic diffuse mesaortitis" by Sproul and Hawthorne (1937), and "diffuse granulomatous aortitis" by McMillan None of these names is satisfactory Until more is known of the nature of the disease it seems likely, as suggested by Harrison, that the terms "giant cell arteritis" and "temporal arteritis" will both be used, the former term emphasising the dominant histological feature and the latter the dominant clinical feature of the disease

## Fatal cases—review of literature

Death occurred in 27 of the recorded cases of giant cell arteritis The findings in these cases are summarised in the accompanying table Post mortem examination was performed on 16 cases No post mortem details were published in the case reported by Chasnoff and Vorzimer (1944), no histology was performed in Robertson's (1947) case, and in a further case, Jennings (1948), post *mortem* examination was limited to the head The twelve cases previously recorded

Table

*Summary of 27 fatal cases of giant-cell arteritis*

| Authors | Sex and age | P.M. | Cause of death | Arteries affected (histologically confirmed) |
|---|---|---|---|---|
| 1. Barnard (1935) . . | F. 63 | + | Erysipelas | Anterior descending and posterior coronary; internal and intracranial carotid |
| 2. Sproul and Hawthorne (1937) Case 1 | M. 76 | + | Septic kidney | Aorta; both common iliacs |
| 3. Sproul and Hawthorne (1937) Case 2 | M. 50 | + | Cardiac infarct | Aorta; iliac; carotid |
| 4. Gilmour (1941) Case 1 | F. 23 | + | Ruptured subclavian aneurysm | Aorta; both subclavians and internal carotids |
| 5. „ Case 2 | F. 59 | + | Cerebral infarct | Aorta; both internal carotids |
| 6. „ Case 3 | M. 63 | + | Cerebral infarct | Aorta; both internal carotids; left external carotid |
| 7. „ Case 4 | F. 64 | + | Post operative | Aorta; innominate; subclavian; common carotid; common iliac |
| 8. Sproul (1942) . . | M. 68 | + | Cardiac infarct | Carotid; innominate; subclavian; pulmonary; cœliac; mesenteric; renal; iliac |
| 9. Post and Sanders * (1943) | F. 66 | − | Cerebrovascular accident | Both temporals (biopsy) |
| 10. Chasnoff and Vorzimer (1944) | F. 63 | + | Coma | Temporal and internal carotid arteries. No post-mortem details published |
| 11. Broch and Ytrehus (1946) Case 1 | M. 69 | − | Hæmatemesis | ... |
| 12. Cooke *et al.* (1946) Case 1 | M. 66 | − | Cerebrovascular accident | Both temporals (biopsy) |
| 13. Cooke *et al.* (1946) Case 3 | F. 69 | + | Multiple cerebral thromboses | Both temporals; aorta; both femorals; subclavians |
| 14. Cooke *et al.* (1946) Case 6 | F. 73 | + | Cerebral death | Both temporals; aorta; radial; superior mesenteric; femoral; both retinals |
| 15. Curtis (1946) . . | F. 71 | − | Cerebral death | Both temporals (biopsy) |
| 16. Kilbourne and Wolff (1946) | M. 68 | − | Cardiac infarct | Both temporals (biopsy) |
| 17. Robertson (1947) Case 4 | M. 67 | + | Perforated gastric ulcer | No post-mortem histology. Thrombus in subclavian |
| 18. Cohen and Harrison (1947-48) Case 2 | M. 65 | − | Coronary occlusion | Temporal (biopsy) |
| 19. Cole (1948) Case 1 . | M. 68 | − | Coronary occlusion | ... |
| 20. „ Case 2 . | M. 64 | − | Bronchitis | ... |
| 21. „ Case 3 † . | F. 72 | − | Cardiovascular accident | ... |
| 22. Dupont and Heerup (1948) | F. 58 | + | Myocardial degeneration | Aorta; innominate; subclavians; common, internal and external carotids; hypogastrics; cœliac; coronary |
| 23. Jennings (1948) Case 3 | F. 73 | + | Coma | Post-mortem limited to head. Temporal; middle meningeal |
| 24. Bergouignan and Lacrotte (1949) Case 2 | F. 64 | − | Cerebral coma | ... |
| 25. Smith and Greene (1949) Case 3 | F. 81 | − | Coronary occlusion | Both temporals (biopsy) |
| 26. McMillan (1950) . . | M. 58 | + | Cerebral infarct | Aorta; common iliac |
| 27. Cardell and Hanley (Case here reported) | M. 68 | + | Cerebral death | Both temporals; aorta; femoral; both internal carotids; both ophthalmics and their branches; coronaries |

* Reported earlier by MacDonald and Moser (1936-37)

† Reported also by Jennings (1938, Case 2)

in which full post mortem reports are available are considered below. Three of these (Cooke *et al*., 1946, cases 3 and 6) and Sproul (1942) were diagnosed clinically as temporal arteritis. The other nine, though clinically atypical, showed histological lesions of giant cell arteritis.

Barnard (1935) under the title of tuberculous arteritis recorded the case of a woman aged 63 who died of facial erysipelas after an illness characterised by headaches of increasing severity with loss of sight in one eye. Routine post mortem examination revealed changes in the internal carotid and coronary arteries consisting of medial necrosis and a cellular infiltrate including giant cells; no tubercle bacilli were found in the lesions. In the light of cases later reported, it is probable that this was an example of giant cell arteritis. Sproul and Hawthorne under the title of chronic diffuse mesaortitis recorded two cases, both men, one aged 76 dying of a suppurative urinary tract infection, and the other aged 50 dying of cardiac infarction following recurrent attacks of precordial pain for one year. Histological lesions of giant cell arteritis were found, in one case in the aorta and common iliac arteries, and in the other in the aorta, iliac and carotid arteries. The temporal arteries were not involved clinically and were not examined at necropsy.

Gilmour under the title of giant cell chronic arteritis recorded four necropsy cases. The first was of a woman aged 23 who had been ill for six months with hot sweats and giddiness, after an influenza like onset. She died of a ruptured subclavian aneurysm. Although the case was clinically atypical, the histological lesions described in the aorta, internal carotid and both subclavian arteries are typical of giant cell arteritis. The age is unusual, but Meyers and Lord (1948) reported a clinically typical case in a woman of 22. In addition, Roberts and Askey (1948, case 2) reported the case of a woman of 25, though the clinical picture was rather atypical and no biopsy was performed. Gilmour's second case was a woman aged 59 who had had headaches and loss of weight for five months following an influenza like illness. She died of a cerebral infarct and necropsy showed giant cell arteritis of both internal carotid arteries and the aorta, though no giant cells were seen in the aorta. The third case, a man of 63, died of a cerebral infarct nine months after an influenza like illness which was followed by headache, mental inertia and failing vision. Lesions of giant cell arteritis were found in the aorta, the left external carotid artery and both internal carotid arteries. No giant cells were seen in the aorta or external carotid artery. Gilmour's fourth case, a woman aged 64 who had been unwell for 26 months following "influenza", died after an operation for intestinal obstruction. At necropsy, lesions which were interpreted as a healing stage of giant cell arteritis were found in the aorta, innominate, subclavian, common carotid and common iliac arteries. No giant cells were seen in any of these vessels.

Sproul recorded the necropsy findings in a man aged 68, with clinically typical temporal arteritis without any ocular complications, who died of ischæmic heart disease. The characteristic lesions were found in the carotid, innominate, pulmonary, cœliac, mesenteric, renal and iliac arteries. The temporal and cerebral vessels were not examined and there is no mention of the histological appearance of the coronary arteries. Cooke *et al*. recorded two necropsies in a series of seven clinically typical cases of temporal arteritis. The first, a woman aged 69 with bilateral blindness, died of multiple cerebral thromboses. Lesions of giant cell arteritis were found in the aorta, both temporal, both femoral and both subclavian arteries. No giant cells were present in the subclavian arteries. The second case, a woman aged 73 with bilateral blindness, died from cerebral causes. Lesions of giant cell arteritis were found in the aorta, and in the radial, superior mesenteric, femoral, both temporal and both retinal arteries. Giant cells were not present in the radial, mesenteric and femoral arteries. Dupont and Heerup (1948) recorded the case of a woman aged 58 who had general malaise and fatigue for twelve months, and for three months a febrile illness with pain in the back, loins, neck and in front of the left ear. There was no temporal artery swelling. Death was due to hypostatic pneumonia and myocardial degeneration. Typical lesions of giant-cell arteritis were found in the following arteries :—innominate, both subclavians, the right common and both internal and external carotids, both hypogastrics, cœliac, a branch of a coronary, and in the aorta. The temporal arteries were sectioned but no evidence of giant-cell arteritis was found. McMillan, under the title of diffuse granulomatous aortitis, recorded the case of a man aged 58 who died of a cerebral infarct after a six months' history of mild ataxia. Necropsy revealed thrombosis of the right middle cerebral and internal carotid arteries, and in the ascending aorta an intimal tear with partial rupture and dissection. Typical lesions of giant-cell arteritis were found in the aorta and common iliac arteries.

## CASE HISTORY

Mr C. N., a printers' warehouseman aged 68, first came under observation in 1942, having collapsed in the street with a severe attack of substernal pain. In 1936 he had had a similar attack diagnosed as myocardial infarction, but had recovered well and had been active and free from thoracic pain in the intervening years.

Physical examination at that time (8.4.42) showed a shocked, anxious man, with feeble heart sounds, a blood pressure of 96/60 mm., and a slightly irregular bradycardia of 44 per minute. A cardiogram (fig. 1) showed low voltage splintered QRS deflections, some elevation of the RS-T segment in leads II and III, and inversion of T wave in leads I, IV F and IV R, the changes of extensive myocardial infarction. The E.S.R. was 12 mm. in the first hour, and the leucocyte count 15·6 thousand per c.mm.

He spent six weeks in bed without recurrence of substernal pain and appeared to recover well, being discharged on 27th May 1942 free from pain or dyspnœa. He returned to work and for the next seven years remained well except for slight stiffness and pain in the hips and lumbar region which began in 1944.

The final illness began early in July 1949 with vague generalised muscular pains, at first most severe in the neck, right shoulder and the calves. He felt generally ill at this time and had troublesome constipation and anorexia. On the 7th August, roughly six weeks after the onset, pain in the right side of his neck spread up into the right temporal and parietal region where it became excruciating. Within a few days the left temporal region was similarly affected and the generalised muscle aches abated. The temporal pain was a constant ache punctuated by bouts of great severity and was severe enough to prevent sleep. He found the upright posture much more comfortable than recumbency and tried to sleep in that position. Tenderness in the scalp itself had not been noticed, and although almost bald, he had not been aware of any unusual appearance of the vessels of the scalp.' Trismus, lachrymation and rhinorrhœa were not experienced.

On the 8th September he found himself seeing double, but after four days this disappeared suddenly and he then discovered, on covering the eyes alternately, that he was totally blind in the left eye. On the next day he had six attacks of transient loss of sight in the right eye, but the right vision was not materially impaired up to the time he was admitted to King's College Hospital on 14th September.

On examination (14.9.49) he was a very ill man, with an occasional evening rise of temperature to 99·5° F., but the pulse rate rarely exceeded 85 per minute. Both superficial temporal arteries were tortuous, cord-like though not nodular, and actively inflamed. Both vessels were uniformly tender to touch throughout their length. Pulsation was present at first but disappeared when the phase of

TEMPORAL ARTERITIS

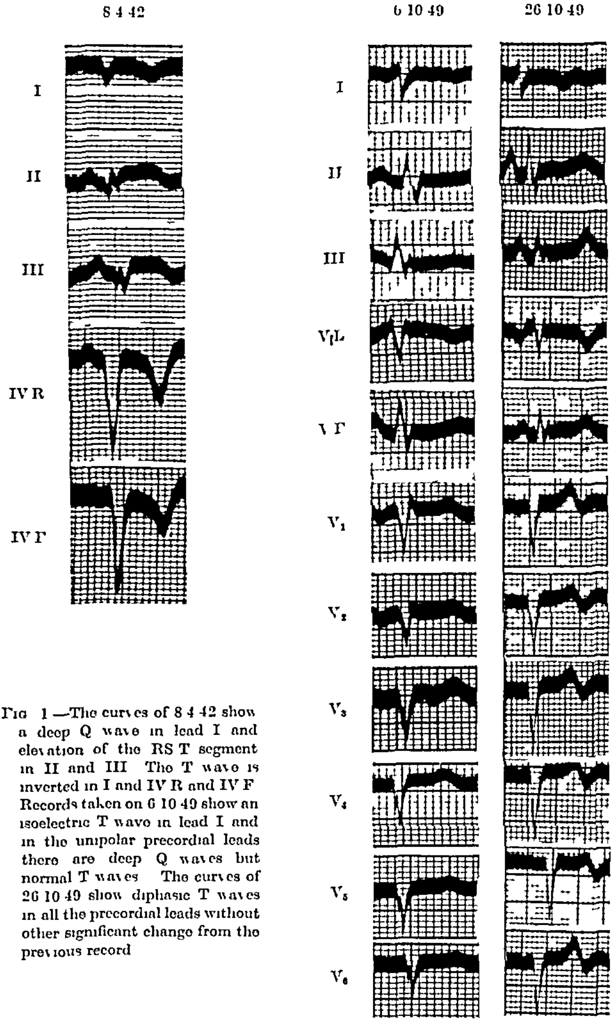

FIG 1 —The curves of 8 4 42 show a deep Q wave in lead I and elevation of the RS T segment in II and III The T wave is inverted in I and IV R and IV F Records taken on 6 10 49 show an isoelectric T wave in lead I and in the unipolar precordial leads there are deep Q waves but normal T waves The curves of 26 10 49 show diphasic T waves in all the precordial leads without other significant change from the previous record

active inflammation subsided in a few days. The other superficial arteries were moderately thickened but none was painful or inflamed

We are greatly indebted to Mr L. H. Savin for the following ophthalmological report: On 10th September the left fundus showed the picture of complete occlusion of the central retinal artery. The right fundus showed a severe arterial spasm in the inferior temporal branch of the central retinal artery, and the veins of both retinæ were much engorged.

Physical examination did not reveal any other abnormal signs. There was some emphysema which masked the apex beat, the heart sounds were normal, and there was no evidence clinically of the myocardial aneurysm subsequently found at post-mortem. The blood pressure was 155/70 mm. Hg. and the urine normal.

The E.S.R. on two occasions was strikingly high, 110 mm and 126 mm. in the first hour. The leucocyte count was 10 6 thousand per c.mm. with 63 per cent. neutrophils and 4 per cent. eosinophils

A cardiogram on 6.10.49 (fig. 1) showed an isoelectric T wave in I, and in all the unipolar precordial leads there was a deep Q wave but the T waves were normal. The appearances suggested past extensive transmural anterior infarction. The transient loss of vision which occurred on 8th September was successfully treated by massage of the globe and inhalation of amyl nitrite. This produced a return to normal of vision and of fundus appearances in the right eye. Two further transient attacks of amblyopia on 18th September were similarly relieved, but on 26th September vision in the right eye was permanently lost. The fundus showed at this time extreme pallor with greatly narrowed calibre of the arteries and arterioles, and the veins showed a remarkable streaming of blood towards the disc. Next day streaming had ceased, and there were sections of empty vein between what seemed to be thrombotic stretches A slow "cattle truck" flow was taking place in the arteries, the blood column progressing in jerks in time with the heart beat. On 29 9.49 a slow flow of blood could be seen in the inferior nasal branches of the central vein. On 1st October arterial spasm was again evident and by then blindness was complete with both fundi presenting the picture of complete central arterial occlusion

Anticoagulant therapy was given using dicoumarol and heparin from 14th to 29th September in the hope of averting further thromboses Satisfactory anticoagulative effect was obtained as judged by prothrombin times but the course of the illness was not affected, indeed thrombosis of the right central retinal artery became complete at the peak of the anticoagulant effect. Nicotinic acid also appeared ineffective.

Although now totally blind his temporal pain had gone and his general condition was slightly improved He was ambulant by 21st October but four days later developed right frontal headache and vertigo and shortly afterwards severe dysphagia and dysarthria. Weakness of the right facial movements, nystagmus, and a right palatal palsy were apparent and he was considered to have had a cerebral thrombosis, probably in the brain stem A cardiogram on 26 10 49 (fig. 1) showed biphasic T waves in all the precordial leads, but there was no clinical evidence of recent myocardial infarction The dysphagia became very severe, his general condition deteriorated and he died on 26th October 1949, roughly sixteen weeks from the first appearance of the symptoms.

## POST-MORTEM EXAMINATION

A complete examination was made eighteen hours after death. The body was that of a thin elderly man The heart (355 g.) showed an old infarct of the anterior wall of the left ventricle and anterior part of the interventricular septum, with a central calcified aneurysmal area measuring 3·7×2·2 cm., with thinning and fibrosis of the surrounding myocardium, and overlying firm pericardial adhesions. There was another old infarct on the posterior wall of the left ventricle, measuring 2·0×1·1 cm., with marked myocardial thinning, some areas of calcification, and overlying fine pericardial adhesions. The coronary arteries showed extensive atheroma with severe narrowing of the lumen in several areas. The anterior descending and circumflex branches of the left coronary artery both divided into two branches immediately proximal to the anterior and posterior myocardial infarcts respectively. The aorta, its major branches and the basal cerebral arteries showed a moderate degree of atheroma. The temporal arteries were not enlarged, and no lumen was visible on the cut surface of either vessel. The lungs (R. 740 g., L. 480 g.) showed moderate congestion and œdema, and there were fine bilateral pleural adhesions. The liver (1360 g.) showed extensive diaphragmatic adhesions and mild chronic venous congestion. The spleen (135 g.) showed extensive perisplenitis. The brain (1300 g.) was macroscopically normal. There were no significant findings in the other organs.

## HISTOLOGY

The left and right temporal arteries both showed the typical picture of giant-cell arteritis (figs. 2, 3 and 4). The lumen was reduced to a mere slit by marked intimal thickening. There was no thrombosis. The inner zone of the intima was composed of cellular fibrous tissue of loose texture with mucoid material between the cells and a scanty cellular infiltration of macrophages and lymphocytes. The outer zone of the intima was composed of vascular connective tissue with a heavy cellular infiltration of macrophages and lymphocytes, a lesser number of plasma cells and very occasional neutrophil and eosinophil leucocytes. The line of demarcation from the media was indistinct, large segments of the internal elastic lamina were destroyed and the surviving portions showed fragmentation and reduplication. The maximal lesion was seen in the media, the whole circumference of which was involved but not uniformly. There were areas of coagulative necrosis and a heavy cellular infiltration similar to that of the outer intimal zone. Multinucleated giant cells of both the foreign body and Langhans' types were present, chiefly in relation to the fragmented internal elastic lamina. The adventitia showed a cellular infiltration similar to that of the media but of lesser degree, and no giant cells were present. The cellular infiltration extended into the periadventitial connective tissue, and the vasa vasorum showed minimal cellular cuffing.

An essentially similar appearance was seen in the following arteries: right and left ophthalmic, left retinal, right and left internal carotid at the site of origin of the ophthalmic artery, the two terminal branches of the left anterior descending coronary, the two terminal

## TEMPORAL ARTERITIS

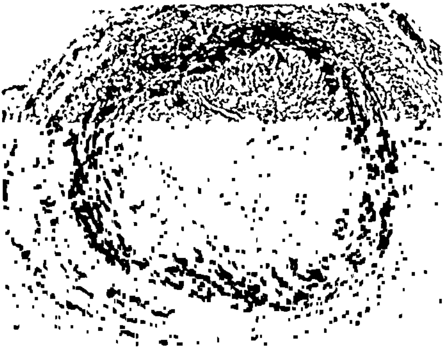

FIG. 2.—Left temporal artery. Gross thickening of intima, with mucoid change in inner zone. Hæmatoxylin and eosin. ×30

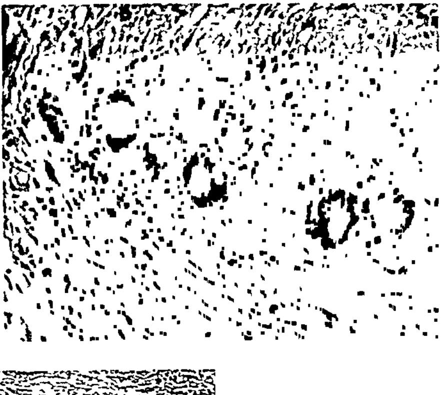

FIG. 3.—Left temporal artery. Outer zone of intima and media. Cellular infiltration and giant cells. H. and E. ×175

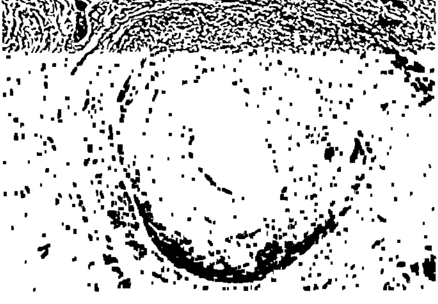

FIG. 4.—Right temporal artery. Intimal thickening and cellular infiltration of wall. H. and E. ×50.

branches of the circumflex coronary and the right femoral In the right ophthalmic artery the lumen was much reduced in size by intimal thickening and an organising thrombus was present in the lumen In the deeper part of the intima there was a fine granular deposit giving a positive iron reaction with Prussian blue There was a small focus of medial necrosis showing a fine granular deposit resembling calcium, but not staining with alizarin red There were no changes in the wall of the right retinal artery, but an organising thrombus was present in the lumen In the left ophthalmic artery the cellular infiltration, including multinucleated giant cells, extended out into the periadventitial tissue (fig 5) The proximal portion of the left retinal artery showed typical changes of giant-cell arteritis The lumen was reduced to a slit, but no thrombus was present Sections of the eyes showed gross degeneration of the optic nerves, the left more advanced than the right In addition, on the right side several of the posterior ciliary arteries showed marked intimal thickening and diffuse cellular infiltration of all coats of the vessel wall with macrophages, lymphocytes and occasional plasma cells In one artery very occasional giant cells were present The right carotid artery showed giant cell arteritis involving half the circumference of the vessel and atheromatous changes in the other half There was little decrease in the size of the lumen The left carotid artery showed typical lesions of giant-cell arteritis involving the whole circumference of the vessel and the lumen was reduced to about half its normal size One branch of the left anterior descending coronary artery had a large atheromatous plaque involving half the circumference of the vessel and typical giant cell arteritis in the other half, the lumen of the vessel being almost completely occluded In the other three affected coronary arteries the lesion involved the greater part of the circumference with gross diminution in the size of the lumen (fig 5) The lumen of the right femoral artery was moderately diminished The thickened intima was composed of loose-textured cellular fibrous tissue with a scanty cellular infiltration of macrophages and lymphocytes There were patchy areas of destruction of the internal elastic lamina in relation to which occasional giant cells were found The media showed occasional small foci of degeneration and there was a diffuse cellular infiltration of macrophages and lymphocytes There was some thickening of the adventitia

Sections were taken of the ascending and descending arch, the descending thoracic and abdominal portions of the aorta As the findings were somewhat equivocal, the aorta was divided into five portions of approximately equal length, and from each portion a longitudinal strip was taken and treated by the " Swiss roll " technique (Magnus, 1937), so that the whole length of the aorta was examined Throughout the aorta there were occasional atheromatous deposits In addition and independently there were patchy areas of chronic medial degeneration The internal elastic lamella was absent and there was slight fibrosis in the inner part of the media with some collagen formation and, in some areas, separation of the elastic fibres. There was patchy cellular infiltration with lymphocytes, macrophages and plasma cells and a few capillaries were present. The intima showed patchy sclerosis and a scanty infiltration with lymphocytes, macrophages and plasma cells. The adventitia showed little apart from occasional cellular cuffing of the vasa vasorum.

The following vessels showed no evidence of giant-cell arteritis :—innominate ; left subclavian ; right and left common carotid ; origin of right external and internal carotid ; terminal portion of right and left internal carotid ; superior and inferior mesenteric ; right renal, brachial, radial and dorsalis pedis ; right coronary and its three major branches ; left coronary and proximal portions of its anterior descending and circumflex branches ; basilar ; right and left anterior, middle and posterior cerebral ; and right retinal. The right coronary artery and its three major branches showed moderate atheroma with areas of calcification and slight to moderate decrease in size of the lumen. The left coronary artery showed mild atheroma with only slight diminution in size of the lumen ; the proximal portions of the anterior descending and circumflex branches showed severe atheroma with gross intimal thickening and only a minute lumen and, in addition, an old recanalised thrombus was present in the circumflex branch.

There was no abnormality of the small vessels of the kidneys, suprarenals or pancreas. Sections of the heart and liver confirmed the macroscopic findings.

## DISCUSSION

Considering the twelve recorded cases in which full post-mortem reports are available together with the present case, the frequency with which involvement of individual arteries has been recorded is as follows :—aorta—eleven times ; internal carotid—six times ; external or common carotid, subclavian and iliac—five times ; temporal, coronary, innominate and femoral—three times ; cœliac, mesenteric and ophthalmic or its branches—twice ; hypogastric, radial and pulmonary—once. These figures do not give a true picture of the incidence of giant-cell arteritis in the arterial system, as all the vessels listed above were not examined in each case, but they show that giant-cell arteritis is a generalised disease and some interesting points emerge from an analysis of the ill-effects of the lesions.

Of special interest in the present case is the finding of lesions in the coronary and ophthalmic arteries. The only coronary arteries affected by giant-cell arteritis were those supplying the two infarcted areas of myocardium. It is tempting to suggest that giant-cell arteritis was responsible for the vascular closure producing myocardial infarction. However, the two infarcts occurred seven and thirteen years respectively before the fatal illness and it appears unlikely from our

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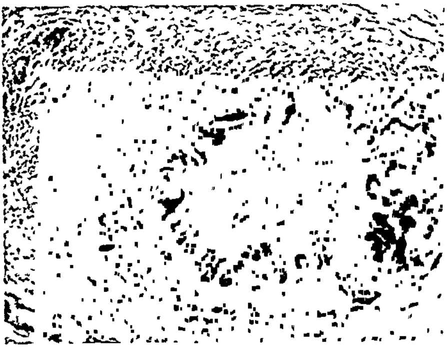

FIG. 5.—Left ophthalmic artery. Typical lesion, together with periadventitial giant cells. H. and E. ×75.

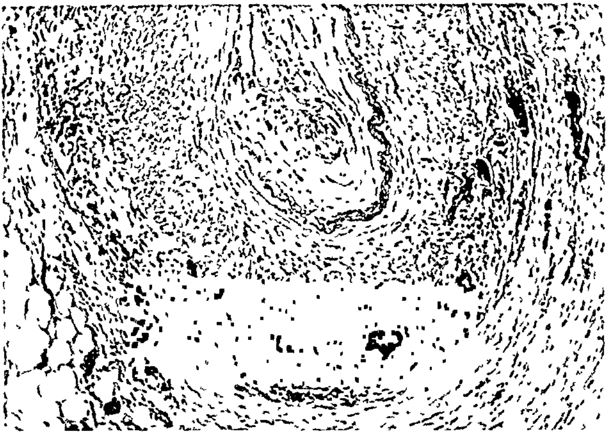

FIG 6 —Circumflex branch of left coronary artery. Intimal thickening and cellular infiltration of wall, including giant cells H. and E. ×100.

present knowledge of this disease that active lesions can persist for such a long period. Furthermore, in both instances the more proximal portion of the vessel supplying the infarcted area showed gross atheroma with almost complete obliteration of the lumen. It seems more probable therefore that the atheromatous lesions were responsible for the myocardial infarcts. Barnard observed typical lesions in both the anterior descending and posterior coronary arteries of his case, but there was no ischæmic heart disease. Dupont and Heerup noted a lesion in a branch of a coronary artery together with degenerative changes in the heart muscle. Cooke *et al.* (case 6) noted marked lymphocytic and plasma-cell infiltration of the adventitia and media of the coronary arteries, and the heart showed patchy degeneration of the muscle fibres and well-marked replacement fibrosis. Though these findings are rather indefinite it is suggested that giant-cell arteritis may be a rare cause of coronary artery obstruction and it may be noted that ischæmic heart disease was the cause of death in 7 of the 27 recorded fatal cases of giant-cell arteritis.

In the present case, vascular obstruction due to the lesions of the ophthalmic arteries and their branches was clearly responsible for both the optic atrophy and the retinal changes observed clinically. Involvement of the ophthalmic artery or its branches is common in this disease for, in the 137 recorded cases, 52 have had symptoms referable to the eyes and permanent blindness of one or both eyes resulted in 36 cases. The only other histological evidence of such involvement is recorded by Cooke *et al.* (case 6) where both retinal arteries showed typical lesions of giant-cell arteritis with obliteration of the lumen by an organising thrombus.

In view of the fact that 12 of the 27 recorded fatal cases of giant-cell arteritis died from cerebral vascular disease, occlusive lesions in the internal carotid and cerebral arteries would be expected. In the present case the immediate cause of death appeared, on clinical grounds, to have been a cerebral vascular lesion. No structural changes were observed in the brain but considerable occlusive changes due to giant-cell arteritis were present in the left internal carotid artery which may have been responsible for the right-sided paretic signs observed in the two days preceding the patient's death. In two of Gilmour's cases (cases 2 and 3), lesions of the internal carotid artery were associated with thrombosis and a cerebral infarct, and in McMillan's case internal carotid-artery thrombosis and a cerebral infarct were present but the histological lesion in the vessel was equivocal.

Although lesions in the aorta appear to be common they produced ill-effects in only one case. McMillan reported aortic lesions associated with partial rupture and limited dissection of the aorta. In two further cases aneurysm formation has been reported. Gilmour (case 1) recorded a death from rupture of an aneurysm of an affected subclavian artery, and Crosby and Wadsworth (1948, case 2) described a dissecting aneurysm in a biopsy specimen of a temporal artery.

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# THE EFFECT UPON THE GROWTH OF ROUS SARCOMA OF A DIET DEFICIENT IN VITAMIN E

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(Plates XCVIII-C)

In the course of an investigation into the effect of Rous sarcoma upon the endocrine organs of the tumour-bearing host, marked degenerative changes were found in the testes of the cocks and comparatively slight changes in the ovaries of the hens. This suggested the possibility that the tumour might render the birds deficient in vitamin E. The preen glands of the tumour-bearing cocks and hens also showed degenerative changes (Cater, 1951). Experiments were therefore planned to find if a diet deficient in vitamin E would influence the growth of Rous sarcoma, and whether control birds on a vitamin E-deficient diet would show degeneration of testes and preen gland similar to that found in tumour-bearing cocks fed on a normal diet.

A widespread green exudate was often found in the subcutaneous tissues of birds with Rous chicken sarcoma No. 1. This suggested a similarity with the "exudative diathesis of chickens" as described by Dam (1944), and led to the choice of his basal diet No. 97 for the experiments. Although this diet is deficient in vitamin E, it contains 10 per cent. of cod liver oil and is therefore rich in vitamins A and D and the essential unsaturated fatty acids of marine origin.

In the experiments to be described chicks fed on the basal diet No. 97 developed the exudative diathesis and changes in testes and preen gland. They also developed muscular dystrophy with changes similar to those described by Pappenheimer and Goettsch (1934) in the muscles of ducklings fed on a vitamin E-deficient diet.

## Methods

The basal diet No. 97 of Dam used in the experiments contains per 100 g. :—cod liver oil 10 g. ; dried yeast (ether extracted) 10 g. ; casein 15 g. ; sucrose 44·9 g. ; gelatine 8 g. ; gum arabic 5 g. ; McCollum's salt mixture No. 185 5 g. ; and choline 0·1 g., together with supplements of vitamin K substitute and copper, manganese and iodine salts (Dam, Kruse, Prange and Sondergaard, 1948).

Attempts to render cocks of 3-4 months of age vitamin E deficient on this diet failed to produce any of the signs of the exudative diathesis within the experimental period. It was therefore necessary to use day-old chicks, in which this diet produces the exudative diathesis in about 12 days.

Twenty-five day-old cocks were divided into 6 groups of equal weight distribution and treated as detailed in table I. Birds fed on a normal laboratory diet and with or without Rous sarcoma were also available for comparison.

TABLE I

*Details of diet and treatment in an experiment to show the effect of vitamin E upon the growth of Rous sarcoma and upon the histology of the testes and preen gland*

| Group | No. of birds | Diet | Supplement of tocopherol acetate per 100 g. of diet | Injection of Rous sarcoma cells on 17th day |
|---|---|---|---|---|
| 1. N−E | 5<br>(one died) | Basal diet<br>No. 97 of Dam | None | None |
| 2. T−E | 5 | ,, | None | Tumour injected |
| 3. N+E | 5 | ,, | 10 mg. | None |
| 4. T+E | 5 | ,, | 10 mg. | Tumour injected |
| 5. N++E | 1 | ,, | 30 mg. | None |
| 6. T++E | 4 | ,, | 30 mg. | Tumour injected |

Individual weight curves of the birds in all groups were kept and the birds were regularly examined for signs of the exudative diathesis. The vitamin E-deficient groups N−E and T−E showed exudates beneath the skin of the breast, loss of activity and appetite and some slowing of the rate of growth before the 17th day. Group T−E therefore showed signs of vitamin E deficiency before being injected with Rous sarcoma cells.

When 17 days old (15th day of feeding) the birds in groups T−E, T+E and T++E were injected with 0·05 ml. fresh Rous sarcoma hash into the right breast muscles. Palpable tumours were present 4 days later and continued to grow until the birds were killed. Because of the time involved in the post-mortems only half the birds could be killed on the 28th day ; the rest had to be left until the 29th or 30th day. For the sake of uniformity it is desirable to refer findings such as tumour-weight to the same day and it was assumed as a first approximation that tumour growth was proportional to its age in order to estimate the weight of tumour on the 28th day in the birds killed one or two days later.

The birds were killed by cervical dislocation, the tumour was weighed and material was taken from tumour, testes, preen gland, muscle and adrenals. Tissues from controls and tumour-bearing birds were compared by the following histochemical techniques :—

Esterase activity by the method of Nachlas and Seligman (1948-49) ;
Alkaline phosphatase, by the Gomori technique, keeping within the time limits, etc., suggested by Danielli (1946) ;
Acid phosphatase by the Gomori (1941) technique ;
Polysaccharides by the periodic acid-Schiff's reagent method (P.A.S.), (McManus, 1948) ;
Lipids by the prolonged osmic-acid staining technique used by Dalton and Peters (1944-45) for adrenal cortex.

## RESULTS

### *Growth and clinical appearance of the birds*

From 15 days old onwards, the vitamin E-deficient birds were listless, ate poorly, tended to squat or had unsteady gait and neck retraction ; their combs were pale, and œdema, sometimes with green discolouration, could be seen beneath the skin of the breast. This œdema was intermittent. Some birds developed a swelling at the angle of the jaw, and some a swelling of the joints of the legs.

Up to 14 days the weight curves of the different groups showed no significant variation. From this point they diverged ; the vitamin E-deficient groups (N—E and T—E) increased but little in weight, whereas the groups supplemented with alpha tocopherol, 30 mg./100 g. of diet, both with and without tumours (N++E and T++E), grew very rapidly and showed excellent development of feathers. The groups with tocopherol 10 mg./100 g. of diet (N+E and T+E) grew somewhat less vigorously. The vitamin E-deficient birds with tumours (T—E) appeared a little healthier than those without (N—E) until the tumours began to get large. One bird of group N—E died when 18 days old ; the others survived but in very poor condition.

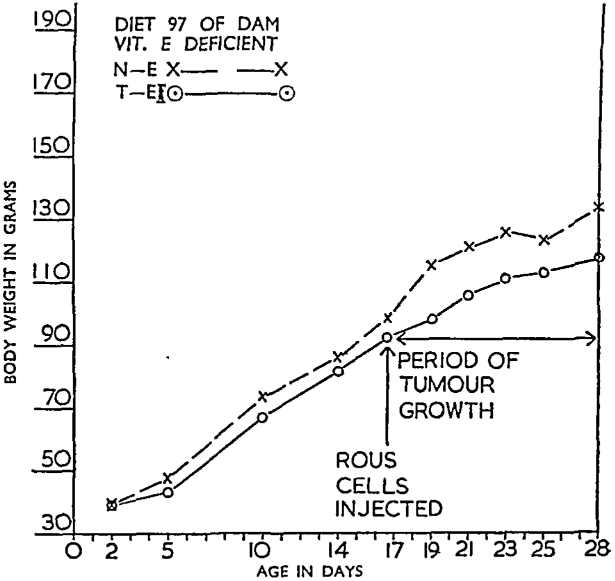


FIG. 1.

### *The growth of the tumour in relation to the growth of the bird*

The rates of growth of the birds in the different groups are shown in figs. 1, 2 and 3.

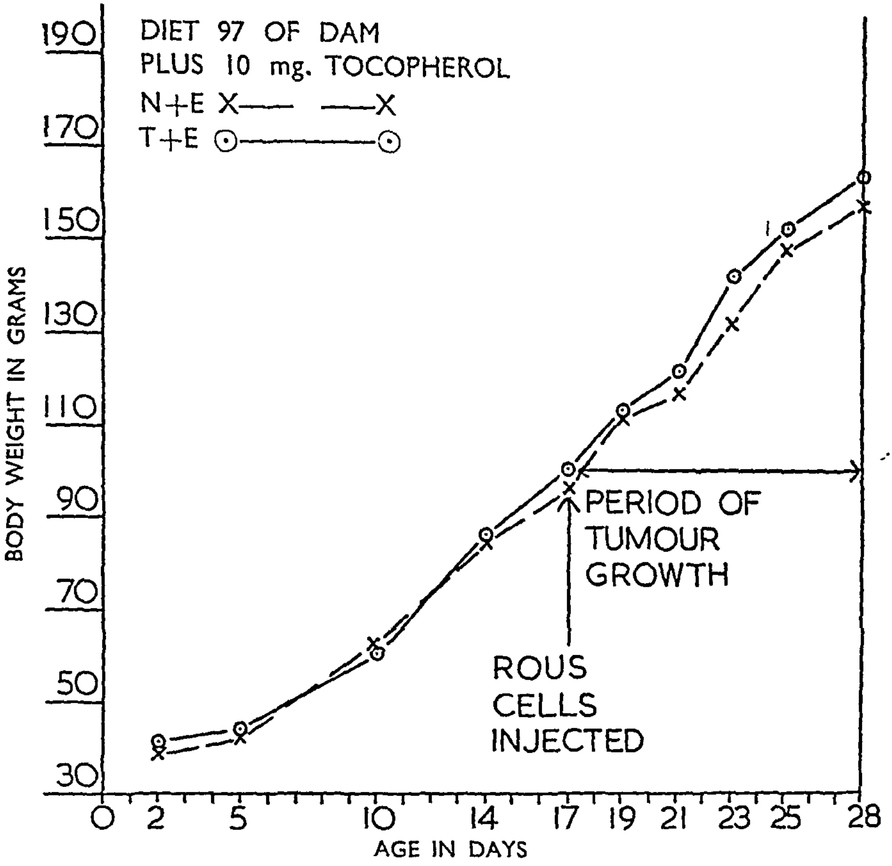

DIET 97 OF DAM
PLUS 10 mg. TOCOPHEROL
N+E X—— ——X
T+E ⊙————⊙
BODY WEIGHT IN GRAMS
190
170
150
130
110
90
70
50
30
0
2
5
10
14
17
19
21
23
25
28
AGE IN DAYS
PERIOD OF TUMOUR GROWTH
ROUS CELLS INJECTED

FIG. 2.

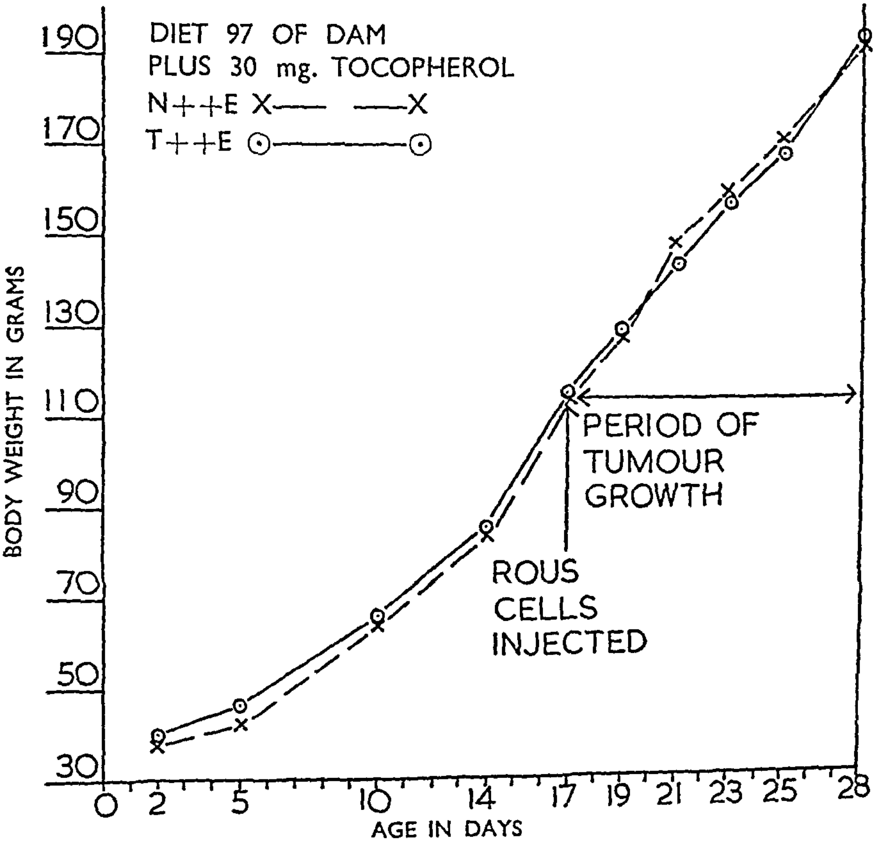

DIET 97 OF DAM
PLUS 30 mg. TOCOPHEROL
N++E X—— ——X
T++E ⊙————⊙
BODY WEIGHT IN GRAMS
190
170
150
130
110
90
70
50
30
0
2
5
10
14
17
19
21
23
25
28
AGE IN DAYS
PERIOD OF TUMOUR GROWTH
ROUS CELLS INJECTED

FIG. 3.

The absolute size of the tumours was greatest in group T+E and least in T++E, but the difference was not statistically significant. The size of the tumour relative to the body weight was greatest in group T−E and least in group T++E, with group T+E occupying an intermediate position (table II).

TABLE II

*Average weight of tumour in relation to average weight of host*

| Group | Average tumour weight | Average weight of birds on 28th day | Tumour weight as percentage of body weight |
|---|---|---|---|
| T−E | 13·7 g. | 118 g. | 11·6 |
| T+E | 16 0 g. | 165 g. | 9·7 |
| T++E | 11·7 g. | 192 g. | 6·1 |

The vitamin E-deficient birds put on very little weight after injection with the tumour cells on the 17th day. The weight of the tumour accounted for 51·5 per cent. of the total increase of weight from the 17th to the 28th day in group T−E, for 25·0 per cent. in group T+E and for 15·5 per cent. in group T++E. The marked retardation of growth on the vitamin E-deficient diet thus failed to retard the growth of the Rous sarcoma; in fact the post-mortem appearances suggested that the tumour was growing relatively better in the vitamin E-deficient birds.

### *Macroscopic appearances of Rous sarcoma*

The naked-eye appearance of the Rous sarcoma in the groups fed the diet supplemented with tocopherol was of the usual type. The tumours were confined to the muscle or were just beginning to fungate along the track of the needle. The skin was not adherent or infiltrated. In sharp contrast, all the tumours in the vitamin E-deficient group showed unusual features. They were white, fleshy, and without necrosis or hæmorrhage. There were numerous, fine, local, secondary deposits in the œdema fluid over both pectoral muscles and down the right leg. The skin over the tumour was adherent, thickened and infiltrated. These appearances suggested that there were factors favouring the local spread of Rous sarcoma in the birds with the exudative diathesis.

### *Post-mortem signs of vitamin E deficiency*

Group N−E showed pale, watery-looking breast muscle in contrast with the red muscle of group N++E. Two out of five of the birds in group N+E showed faint, pale streaks in the muscle, suggesting that the breast muscle might be affected in the vitamin E-deficient birds and prompting histochemical examination of muscle. One bird in group N—E had a zone of red, translucent muscle in the leg. The scanty fat was yellow-brown in colour and subcutaneous œdema had been present in each of the vitamin E-deficient birds at one time or other between the 15th and 28th days. Two out of four of group N—E still showed œdema at post-mortem examination. Where the œdema had subsided the skin was adherent to the muscle.

It is interesting to note that the presence of the Rous sarcoma in the groups supplemented with alpha tocopherol caused œdema in relation to the tumour. Four out of five birds in group T+E showed more or less green œdema and three out of four in group T++E showed some œdema surrounding the tumour.

All preen glands were white instead of the usual buff colour, possibly owing to the absence of carotenoids from the synthetic diet. The preen glands of groups T+E and N—E were smaller than those of group N+E, and those of group T—E were yet smaller.

The testes of groups N—E and T—E were small, misshapen and sometimes yellowish in colour.

## *Histochemical results*

*Preen gland.* Histochemical examination of the preen glands by the techniques for esterase, acid phosphatase, glycogen and lipids showed that increasing degrees of activity were caused by Rous sarcoma, vitamin E deficiency and vitamin E deficiency-plus-Rous sarcoma, leading in the last group to a marked degree of work atrophy. This is illustrated in figs. 4, 5, 6 and 7, which show the preen glands of the four key groups stained for esterase activity. Cater and Lawrie (1950) described two parts of the preen gland, an outer sebaceous and an inner glycogen zone. In fig. 4 the lower half of the section shows the glycogen zone with its cells staining for esterase. The upper half of the section shows the sebaceous zone with little activity in the cells and scanty secretion in the tubules. The preen gland from group T+E (fig. 5) shows considerable esterase activity in the cells and more secretion in the tubules of the sebaceous zone; the secretion stains strongly for esterase. These changes are even more pronounced in group N—E (fig. 6). In group T—E the process of work exhaustion is almost complete and the cells of the tubules in the sebaceous zone have broken down to form large masses of secretion. There is great reduction in the thickness of the walls of the tubules and the tubules are much shorter in length so that the whole organ is thinned. The tubules in the glycogen zone also appear to be undergoing atrophy (fig. 7).

The other histochemical techniques give results which are consistent with the above interpretation. Group T++E shows changes similar to group T+E although less marked.

*Testes.* The testes in group N+E show tubules lined by an orderly

## PLATE XCVIII

FIG. 4.—Preen gland of a normal cock fed on diet 97 of Dam+10 mg. tocopherol/100 g. diet (group N+E), stained for esterase activity and lightly counterstained with hæmatoxylin. The cells of the glycogen zone stain well but there is little activity or secretion in the sebaceous zone shown in the upper half of photograph. × 60.

FIG. 5.—Preen gland of a group T+E bird fed on diet 97 of Dam+10 mg. tocopherol/100 g. of diet and injected with Rous sarcoma cells on the 17th day. At post-mortem the tumour constituted 10 per cent. of the body weight. Esterase activity is considerable in the cells and secretion of the sebaceous zone. Esterase stain. × 60.

FIG. 6.—Preen gland of a group N−E bird fed on vitamin E-deficient diet 97 of Dam. Most of the cells in the tubules of the sebaceous zone have turned into secretion. Esterase stain. × 60.

FIG. 7.—Preen gland of a group T−E bird fed on vitamin E-deficient diet 97 of Dam and injected with Rous sarcoma cells on the 17th day. At post-mortem the tumour was 18 per cent. of the body weight. The gland wall is thinned and the degenerate tubules of the sebaceous zone are filled with secretion which stains for esterase. Esterase stain. × 60.

ROUS SARCOMA IN VITAMIN E DEFICIENCY

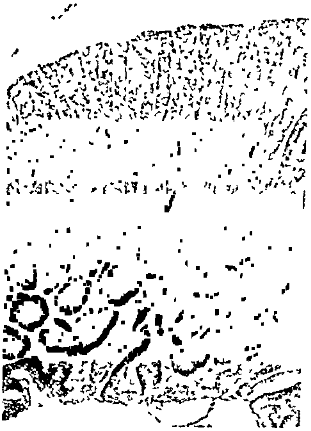
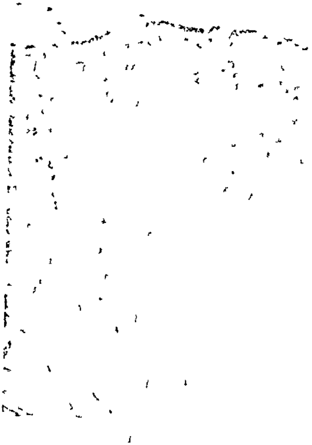


FIG. 4.



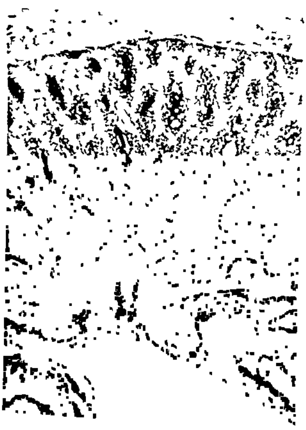


FIG 6

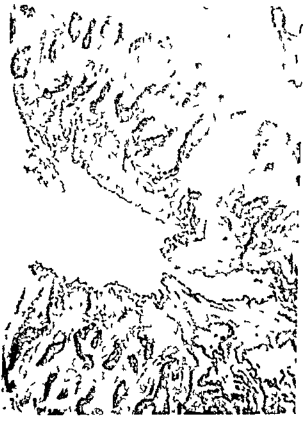


FIG 7

# Plate XCIX

Fig. 8.—Testis of a group N+E bird. The testis cords are tubular and are lined with a single layer of cells. The interstitial cells are moderate in number. Alkaline phosphatase, 2 hrs.' incubation—neutral red. × 255.

Fig. 9.—Testis of a group T+E bird. Rous sarcoma constituted 16 per cent. of the body weight. The cells of the testis cords are multiplying and forming a double layer. The interstitial cells are increased in number. Alkaline phosphatase, 2 hrs.—neutral red. × 255.

Fig. 10.—Testis of a group N−E bird. The testis cords are more than one cell thick and are desquamating into the lumen. The interstitial cells are increased in number. Alkaline phosphatase, 2 hrs.—neutral red. × 255.

Fig. 11.—Testis of a group T−E bird. Rous sarcoma constituted 19 per cent. of the body weight. The testis cords are 2 or 3 layers thick. Strands of interstitial cells are present but are disintegrating. Alkaline phosphatase, 2 hrs.—neutral red. × 255.

ROUS SARCOMA IN VITAMIN E DEFICIENCY

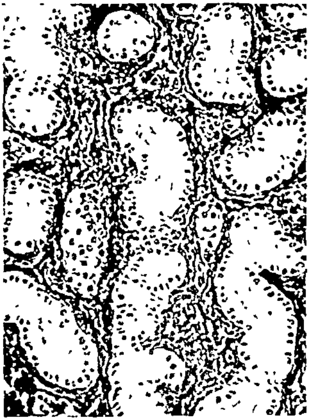


FIG. 8.

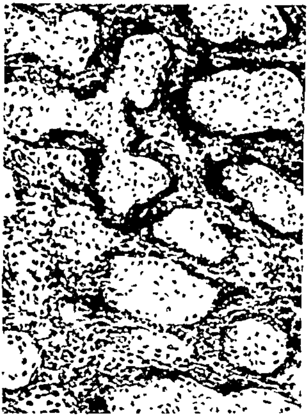


FIG. 9.

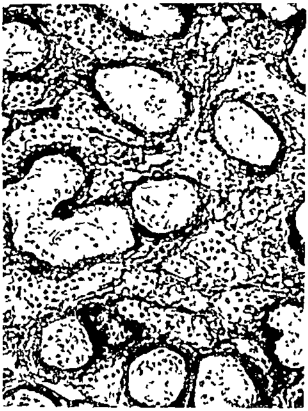


FIG. 10.

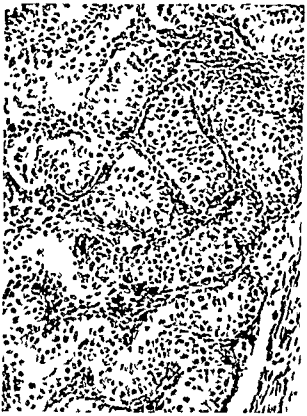


FIG. 11.

layer of cells, one cell thick, surrounding a moderate lumen. The tubules are enclosed by a basement membrane which stains well for alkaline phosphatase, as do the surrounding oval-shaped cells of the stroma. A moderate number of bands of interstitial cells stand out because of their unstained cytoplasm (fig. 8, alkaline phosphatase, 2 hrs.' incubation).

In the testes of group T+E the tubules are more disorderly and the layer of cells lining the tubule is often two cells thick. The numbers of interstitial cells are increased (fig. 9).

The vitamin E-deficient group N—E shows the same type of change. One tubule in fig. 10 shows a mass of desquamating cells in the lumen. The increase of interstitial cells is noteworthy.

In group T—E the process is more advanced, the tubules show two or more layers of cells and the interstitial cells in certain places are undergoing disintegration.

The tendency for the Rous sarcoma and the vitamin E-deficient diet to cause precocious development of the testes in these month-old birds is interesting in view of the retardation of body growth in groups N—E and T—E. It suggests that the desquamation of spermatids and spermatocytes into the lumen of the testis tubule in the 3- to 5-month-old birds with Rous sarcoma (Cater) might be due to a premature attempt to produce germ cells, an uncontrolled overactivity leading to degenerative changes.

The sections stained with osmic acid confirm the increase in the interstitial cells in the vitamin E-deficient group and the tumour-bearing birds. In these groups the individual interstitial cells show much larger droplets of lipid than in the controls.

*Histochemistry of the dystrophic muscle of the vitamin E-deficient chicks.* The vitamin E-deficient chicks of group N—E show marked degenerative changes in the muscle of the breast and legs. Degenerating muscle fibres are present, some of which show wide separation of their transverse striations as if the weakened muscle had been overstretched (fig. 13). The degenerating muscle fibres are separated by wide zones of inflammatory cells, which stain heavily for alkaline phosphatase (fig. 12). This distinguishes them from Rous sarcoma cells, which stain poorly.

Some of the acetone-fixed sections show granular material in degenerating muscle fibres. This stains black by the acid phosphatase method both with and without substrate. As it gives no Prussian-blue reaction for iron, it may be the brown pigment which Moore and Wang (1943) described in dystrophic muscle.

Some of the degenerating muscle fibres show fine P.A.S. positive granules which outline the transverse striations (fig. 14). These no longer stain after incubation for half an hour with salivary amylase and are therefore glycogen.

It is interesting to note that in or near the invading edge of the Rous sarcoma there are patches of degenerating muscle fibres which stain positive with P.A.S. These changes occur not only in groups T+E and T++E but also in birds fed on a normal diet (see fig. 15). These degenerating muscles no longer stain after treatment with salivary amylase, so they contain free glycogen. The droplets of polysaccharide seen in the Rous sarcoma cells are not affected by salivary amylase.

### *Vitamin E and hæmolysis*

Gyorgy and Rose (1949) have shown that the washed red cells from vitamin E-deficient rats are rapidly hæmolysed by dialuric acid. The hæmolysis can be prevented by tocopherol, especially if the red cells are incubated with this substance.

Because of the time involved in the post-mortem examinations, it was possible to test the blood of only one or two samples from each group of chicks for hæmolysis by dialuric acid. Hæmolysis occurred in the chicks on the basal diet No. 97 but only after the mixture of washed red cells and dialuric acid had stood at room temperature for some hours. It was thought that the nuclei of the red cells in birds might make them more robust. The results were consistent with the rest of the findings of the experiment.

### *Histochemistry of Rous sarcoma*

The findings were similar in all respects to those recorded in a previous communication (Cater), with the additional observation that the tumour does not stain by the esterase technique.

## DISCUSSION

It is widely held that tumours grow best in rapidly growing animals. Little, Oleson and SubbaRow (1948) found that the reduction of the caloric intake to two-thirds depressed the growth of Rous sarcoma. In my experiments, the vitamin E-deficient birds ate badly and grew slowly in comparison with the birds whose diet was supplemented with tocopherol; yet they grew tumours, the absolute weights of which were about the same as in the control group and the tumour weight relative to the weight of the bird was greater than in the control group. Either the growth of Rous sarcoma was independent of the presence or absence of vitamin E in the diet, or taking into account the small size and poor rate of growth of the vitamin E-deficient birds the tumour grew relatively better in the deficient birds. The local spread of the tumour in these chicks supports the latter view.

Testicular degeneration can result from vitamin E deficiency (Evans and Burr, 1927; Adamstone and Card, 1934) or from vitamin A deficiency (Mason, 1933) or vitamin A excess (Cormier, 1948) and from lack of the essential fatty acids (Evans, Lepkovsky and Murphy, 1934). Diet No. 97 of Dam is rich in vitamins A and D and unsaturated fatty acids and deficient in vitamin E, and is therefore likely to induce a

## PLATE C

FIG. 12.—Disintegrating muscle fibres, separated by wide zones of inflammatory cells from a vitamin E-deficient chick. Alkaline phosphatase, 24 hrs'. incubation—neutral red. × 255.

FIG. 13.—Disintegrating muscle fibres from a vitamin E-deficient chick showing the transverse striations widely separated. Prolonged osmic acid staining. × 255.

FIG. 14.—The same chick as shown in figs. 12 and 13. One disintegrating muscle fibre stains strongly by the P.A.S. technique and other fibres are surrounded by cells with positively stained granules. P.A.S.—light green. × 255.

FIG. 15.—Rous sarcoma from a chick fed on a normal laboratory diet, showing P.A.S.-positive material present in disintegrating muscle at the edge of the tumour. Some of the tumour cells also stain. P.A.S.—light green. × 255.

# ROUS SARCOMA IN VITAMIN E DEFICIENCY

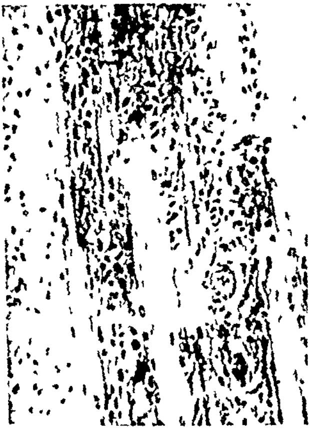


FIG 12

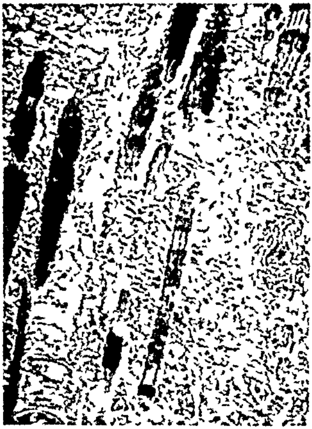


FIG 13

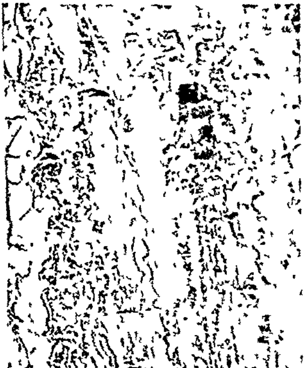


FIG 14

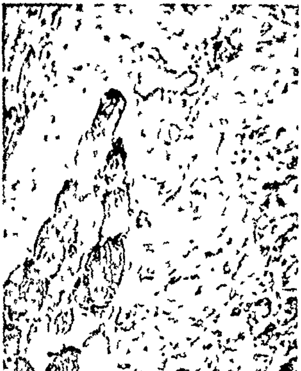


FIG 15

maximum vitamin A and E imbalance. Thus in the exudative diathesis the changes in the testes and preen gland might be due to lack of vitamin E, to excess of vitamin A, to the marine fatty acids, or to a combination of all these factors.

The degeneration of the testes and preen gland in cocks with Rous sarcoma appears similar to that in birds with the exudative diathesis. If the Rous sarcoma caused signs of vitamin E deficiency because it used up the available supplies of vitamin E in its own metabolism, it would be unlikely to grow freely in animals already showing clinical signs of lack of the vitamin. The vitamin E requirements of the Rous sarcoma thus appear to differ from those of the Jensen rat sarcoma recorded by Coke and Reyes (1949). There are at least two possible explanations: firstly, vitamin E may act as a controller of mesenchymal proliferation, as suggested by the presence of abnormal proliferation of the mesenchyme in the vitamin E-deficient chick embryo (Adamstone, 1931) and the Rous sarcoma may inhibit this controlling mechanism; secondly, Rous sarcoma may elaborate a growth-promoting substance with an action similar to that of vitamin A. The two possibilities are certainly not mutually exclusive.

The work of Claude (1935), Jobling, Sproul and Stevens (1937) and Pollard (1939) suggests that the phospholipid fraction is an essential component of the Rous sarcoma virus. The preen gland produces lipids, and Cater and Lawrie (1951) suggested that the sebaceous zone might produce the part of the preen-gland secretion responsible for feather lubrication. Unpublished investigations suggest that phospholipids are present in the secretion of the sebaceous zone of the preen gland. It would be instructive, therefore, to investigate the effect of the lipid fraction of the Rous sarcoma upon this organ.

## SUMMARY

1. The effect of feeding the basal diet No. 97 of Dam (deficient in vitamin E and rich in cod liver oil), with or without a supplement of alpha tocopherol acetate, upon the growth of Rous sarcoma in chicks was investigated. The effects of vitamin E deficiency, Rous sarcoma and vitamin E deficiency together with Rous sarcoma, upon the testes and preen gland were studied histochemically.

2. When Rous sarcoma was inoculated into chicks fed on the basal diet and already suffering from the exudative diathesis, tumour growth was not retarded although the rate of growth of the chicks was conspicuously lowered. The presence of numerous secondary deposits in the vicinity of the primary tumour suggested that the exudative diathesis favoured the local spread of Rous sarcoma.

3. The interstitial cells of the testes were increased by vitamin E deficiency or by Rous sarcoma, and they contained larger drops of lipid than in the controls. In birds with Rous sarcoma or vitamin E deficiency the testicular tubules were lined by two layers of cells

instead of the single layer seen in the normal controls. These changes were more marked in the deficiency-plus-tumour group.

4. Degenerative changes in the preen gland were present in the vitamin E deficiency group and in the tumour group, and were most pronounced in the vitamin E deficiency-plus-tumour group.

5. The results are consistent with the view that vitamin E deficiency causes the same type of change in the testes and preen gland as does Rous sarcoma in birds fed a normal diet. The effects of the deficient diet and the tumour appear to be additive.

6. The histological features of muscular dystrophy were present in the vitamin E-deficient chicks.

I should like to express my thanks to Professor H. R. Dean, Dr T. Moore, Miss E. M. Cruikshank, Dr Kendal C. Dixon and Dr N. R. Lawrie, who have read the manuscript and made many valuable suggestions; to Miss Jean Cook and Miss B. D. Disbrey for technical assistance, and to Mr S. Patman for the photography.

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# THE EXPERIMENTAL PRODUCTION OF PULMONARY ŒDEMA WITH AMMONIUM SALTS, TOGETHER WITH A CLASSIFICATION OF LUNG ŒDEMAS

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(Plate CI)

During 1942 one of us noticed that the intraperitoneal injection of large doses of ammonium chloride gave acute pulmonary œdema in rats. It was not possible at that time to continue the investigation and the matter was forgotten until recent American publications re-awakened our interest and raised our hopes that this particular example might fit into the general plan of study we are developing for pulmonary œdema. A short preliminary account of some of our current work has been given in a lecture by Cameron (1951).

## Methods

We have worked almost wholly with male albino Wistar rats which averaged 150 g. body weight. A few experiments were performed with guinea pigs and mice but since they gave negative results they will not be described. Several male rabbits, 2 kg. body weight, were used for blood pressure observations. Various ammonium salts were injected intraperitoneally or by stomach tube as 6 per cent. solutions in distilled water. With few exceptions the experiments were over within 30 minutes, the animals dying from pulmonary œdema. Animals that recovered were killed with ether or Nembutal either at the end of the experiment or 24 hours afterwards. Protective tests were carried out by intraperitoneal administration of various drugs. Double vagotomy was performed in two stages as described by Cameron and De (1949). The lungs were fixed after death by suspending them by the ligatured trachea in 10 per cent. formol saline for 24-48 hours. After slicing the fixed tissue into small segments it was embedded in paraffin in the usual way and stained with Ehrlich's acid hæmatoxylin and eosin and Weigert's iron hæmatoxylin and van Gieson.

## Results

### *The pulmonary œdema is due to the ammonium ion*

We have produced pulmonary œdema in rats by the intraperitoneal injection of the chloride, carbonate, sulphate, phosphate [$(NH_4)_2HPO_4$], nitrate, acetate, citrate and bromide of ammonium. In all cases we used a 6 per cent. solution; we have made no efforts to find out whether any of these salts are more effective than others. It is obvious from the success we obtained with such a variety of salts that the ammonium ion must be the factor responsible for the œdema. Neither acidosis nor alkalosis can be blamed, for the same reason, but to make sure that this was a fair deduction we put the question to the test by giving large amounts of acid and alkali to rats. No pulmonary œdema appeared under such extreme conditions.

## *The course of the œdema*

The symptoms which develop shortly after the administration of large doses of ammonium chloride, with which most of our work was done, fall into two classes, nervous and respiratory. The former include muscular weakness, hyperexcitability to sound, pain and touch, widespread muscular fasciculations, convulsions and coma in that order. They overlap the respiratory phenomena although the two classes of disturbance may be dissociated and sometimes one class develops without the other. Respiratory symptoms include dyspnœa, stridor and respiratory failure terminating in the final outpouring of frothy fluid or blood from the nostrils.

Pulmonary œdema comes on within 5-15 minutes after injection of ammonium salts. It is preceded by patchy congestion and acute emphysema which rapidly involves both organs. The lungs then have a bright red colour and are fully expanded. Sometimes blood is poured into the air spaces in which case the lungs may feel solid. Free pleural fluid is not found. Microscopical examination shows that the perivascular and peribronchial lymphatics are prominent and then dilated at a very early stage. These changes quickly give place to alveolar œdema, at first localised and regional but soon generalised (figs. 1 and 2). Small areas of hæmorrhage are always present in these patches of œdema and not uncommonly they rapidly become extensive. No simple explanation, such as rupture of capillaries, has been discovered for the hæmorrhage and we are forced to regard it as an extreme instance of permeability change within the capillaries, favoured, perhaps, by the violent respiratory movements which set in at this stage. The resemblance to other forms of acute pulmonary œdema is close and no stage or feature is omitted. The œdema fluid is rich in protein from the beginning. The outcome of ammonium intoxication is thus a strictly localised pulmonary œdema. Apart from dilatation of the right heart we have found no other structural disturbance, though we have paid little attention to the histology of the central nervous system.

In all, we have studied the development of ammonium œdema in 60 rats, 40 of which were males, 20 females. Eight animals died with severe nervous symptoms before there was time for œdema; the incidence of œdema was 87 per cent. both in males and females.

The latter were somewhat more sensitive to ammonium chloride than males, a dose of 7-8 c.c. per kg. body weight of a 6 per cent. solution being effective in the former case as against 9-10 c.c. per kg. of the same solution for males.

### *Absence of hæmodynamic disturbances in the rabbit after intravenous injection of ammonium chloride*

In four rabbits the carotid and right auricular pressures were recorded by the methods used by Cameron and De, with the animals under Nembutal anæsthesia. Varying amounts of 6 per cent. solution of ammonium chloride were injected intravenously, the maximum dose given being 7 c.c. per kg. body weight, either singly or repeated several times. Gross pulmonary œdema did not develop, but convulsions were induced with regularity. Microscopical examination of the lungs from these animals showed distended hilar lymphatics and small to medium-sized patches of œdema throughout the organs. No significant change in arterial and venous pressures occurred during the pre-convulsive phase. With convulsions the right auricular pressure rose rapidly as the carotid pressure fell. We conclude that lesser degrees of pulmonary œdema develop in the rabbit after the intravenous injection of ammonium chloride without hæmodynamic disturbance.

### *The pulmonary œdema is not prevented by double vagotomy nor by cervical sympathectomy*

Five adult male rats, 150-200 g. body weight, were submitted to double vagotomy under light ether and Nembutal anæsthesia, due regard being paid to the precautions mentioned by Cameron and De. Each rat was given 10 c.c. of 6 per cent. ammonium chloride solution intraperitoneally immediately after division of the second vagus nerve. In five control rats a mock operation was performed in which all procedures were gone through except section of the vagi. These, too, received a dose of ammonium chloride intraperitoneally similar to the first group. Fatal pulmonary œdema developed in all ten animals in 5 to 15 minutes.

Division of both cervical sympathetic chains in the lower third of the neck was performed in four male rats, along with mock sympathectomies in four control animals. Pulmonary œdema killed the eight animals within 15 minutes when ammonium chloride was administered in the usual fashion.

### *The œdema can be prevented or modified by various drugs*

We have tried to prevent or modify the pulmonary œdema by giving various drugs to the animals before injecting ammonium chloride intraperitoneally (fig. 3). The results are summarised in the table.

1. *Depression of the central nervous system.* Morphine sulphate, 20 mg. per kg. body weight subcutaneously, prevented pulmonary œdema in nine out of a group of ten rats. Chloralose, 60 mg. per kg. intraperitoneally, prevented œdema in all members of a group of 10 rats. Ten rats were kept under deep ether anæsthesia for periods up to two hours after injection of ammonium chloride. Four which died within an hour showed moderate to severe œdema. Of ten rats given 40 mg. per kg. Nembutal half an hour before administration of ammonium chloride, seven died of severe pulmonary œdema in 25-95 minutes. Each experiment was carefully controlled.

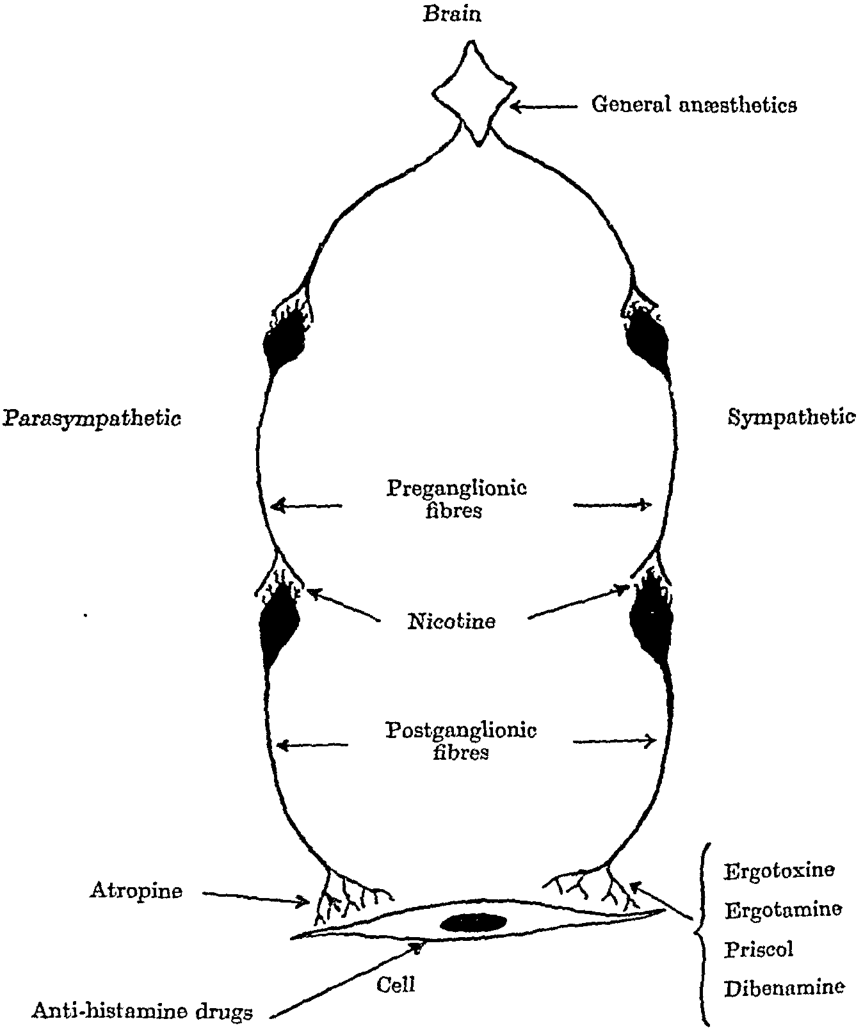

FIG. 3.—Diagram showing sites of action in the nervous system of various drugs used in the prevention of ammonium pulmonary œdema.

2. *Blocking of autonomic ganglia.* Nicotine, which blocks ganglion cells, was injected subcutaneously in 0·5 c.c. doses of 1 : 100 aqueous solution of pure alkaloid into ten rats. This dose gives pronounced

effects for at least half an hour. Ammonium chloride was injected intraperitoneally 15-20 minutes after nicotine. Only two rats showed moderate pulmonary œdema. All five control rats died from severe œdema.

TABLE

*The prevention of ammonium chloride pulmonary œdema by means of various drugs*

| Drug | Interval between administration of drug and ammonium chloride (minutes) | No. of rats treated | No. with pulmonary œdema | Per cent. pulmonary œdema |
|---|---|---|---|---|
| None . . . . . . | 0 | 60 | 52 | 87 |
| Cortisone . . . . . | 20 hours | 5 | 5 | 100 |
| Atropine sulphate . . | 60 and 15 | 20 | 16 | 80 |
| Phenergan . . . . . | 50 | 10 | 8 | 80 |
| Anthisan . . . . . | 30 | 10 | 7 | 70 |
| Nembutal . . . . . | 30 | 10 | 7 | 70 |
| Ether anæsthesia . . | At once | 10 | 4 | 40 |
| Ergotoxine . . . . . | 2 daily doses, 3-5 hours after 2nd dose | 10 | 4 | 40 |
| Cocaine hydrochloride . . | 15 | 10 | 2 | 20 |
| Nicotine . . . . . | 20 | 10 | 2 | 20 |
| Morphine sulphate . . | 40 | 10 | 1 | 10 |
| Chloralose . . . . . | 40 | 10 | 0 | 0 |
| Dibenamine . . . . | 15 | 10 | 0 | 0 |
| Priscol . . . . . | 15 | 10 | 0 | 0 |

3. *Blocking of cholinergic effector cells.* Two intraperitoneal injections of atropine sulphate, each of 0·26 mg. per 200 g. body weight, were given at 60 minutes and 15 minutes before administration of ammonium chloride. Sixteen of twenty rats died within 20-30 minutes of severe pulmonary œdema.

4. *Blocking of adrenergic nerves.* Ergotoxine ethane sulphonate (physiologically tested) was injected subcutaneously on two successive days into ten male rats. Ammonium chloride was administered 3-5 hours after the second dose. Four rats died with severe pulmonary œdema 40-110 minutes after ammonium chloride injection.

Priscol (2 benzyl-4 : 5-imidazoline hydrochloride) was given in doses of 1 c.c. per kg. body weight subcutaneously 5-15 minutes before ammonium chloride to fifteen male rats. None developed pulmonary œdema (fig. 4).

Dibenamine (N,N-dibenzyl-$\beta$ chlorethylamine hydrochloride) was injected intraperitoneally into ten male rats in doses of 24 mg. per kg. body weight 15 minutes before ammonium chloride was given. All of the rats died in about 10 minutes with severe nervous symptoms but none showed pulmonary œdema. Thus striking results were obtained in the prevention of œdema by the use of adrenolytic drugs although none of these suppressed the central nervous disturbances caused by ammonium chloride.

5. *Anti-histamine drugs.* No success can be claimed for Phenergan (25 mg. per kg. body weight subcutaneously) or Anthisan (25 mg. per kg. body weight subcutaneously) given 30 minutes before ammonium chloride.

6. *Other agents.* Intraperitoneal injection of cocaine hydrochloride, 50 mg. per kg. body weight, 15 minutes before ammonium chloride reduced the incidence of pulmonary œdema, only two of a group of ten rats showing œdema. All of a control group of ten receiving the same dose of ammonium chloride showed extensive œdema of their lungs. Through the kindness of our colleague, Dr A. D. Bangham, we were able to inject ammonium chloride into five rats 20 hours after a large dose of Cortisone (5 mg. per rat). Severe pulmonary œdema appeared in all five animals in 5-25 minutes, identical with that developing in control rats given a similar dose of ammonium chloride.

## DISCUSSION

Our experiments show that intoxication with the ammonium ion quickly produces acute pulmonary œdema of a catastrophic type which terminates fatally within 5-30 minutes. The œdema is accompanied by serious disturbances of the central nervous system which, in themselves, may be responsible for death, for we have been able to prevent pulmonary œdema without modifying the severity of the nervous symptoms. The œdema runs through a typical course which is identical with that found with other forms of acute œdema such as the fibrin neurogenic œdema described by Cameron and De. Thus there is histological evidence of increased lymph flow from the lungs, overflow of fluid from the lung capillaries into the air spaces, frequently hæmorrhage on a large scale and acute patchy emphysema. We obtained no evidence of serious hæmodynamic disturbance in animals receiving large intravenous doses of ammonium chloride until the convulsive stage was reached. The high protein content of the œdema fluid suggests that capillary permeability for protein had been increased and we attribute this to the ammonium intoxication.

In an attempt to carry the analysis of this altered capillary permeability a step further we have sought for means of preventing the pulmonary œdema by interrupting connections between the nervous system and the lungs. Some success has attended our efforts for we have succeeded in inhibiting or completely preventing œdema with central nervous system depressants such as morphine and chloralose, by blocking autonomic ganglia with nicotine and adrenergic nerve endings with Priscol and Dibenamine. On the other hand, blocking cholinergic effector endings with atropine or interrupting parasympathetic pre-ganglionic fibres and afferent fibres from the lungs by double vagotomy did not prevent ammonium œdema. Division of both cervical sympathetic chains likewise was without value, nor were anti-histamine drugs such as Phenergan and Anthisan of use

These results suggest that the nervous system plays an important part in the initiation of acute lung œdema due to ammonium ion intoxication. In particular, the sympathetic system seems to be concerned in this process; there is no reason for incriminating the parasympathetic system since its connections with the lungs and cardio-vascular system can be severed without influencing the course of the ammonium œdema. We would suggest that this form of lung œdema be classified as an adrenergic nervous œdema.

Acute pulmonary œdema has been produced in guinea-pigs, rats, cats and rabbits by the oral and intraperitoneal injection of large doses of various ammonium salts (Windle *et al.*, 1946; Koenig and Koenig, 1948*a* and *b*; 1949*a* and *b*; Mackay *et al.*, 1949; and Winter, 1949). American workers have also prevented or inhibited the œdema by prior administration of adrenergic blocking agents (Koenig and Koenig, 1949*b*; Mackay *et al.*, 1949) but not by the interruption of parasympathetic nerve impulses (Koenig and Koenig, 1949*b*) nor by the use of anti-histamine drugs (Mackay *et al.*, 1949; Winter, 1949). Their results are thus in close agreement with ours. Koenig and Koenig (1949*b*) differ in obtaining negative results with morphine and chloralose and they conclude that central nervous system depressant drugs in doses sufficient to induce anæsthesia do not prevent ammonium pulmonary œdema. These workers, too, favour a sympathetic nervous origin for the œdema and show that bilateral adrenalectomy in rats reduces, but does not prevent œdema, that section of the spinal cord at or above the level of the first thoracic vertebra is also effective, but that division of the cervical sympathetic chain does not prevent œdema. Mesencephalic decerebration, too, was effective in rats which survived the operation. Koenig and Koenig point out that hypertension is not a necessary prerequisite for pulmonary œdema since œdema sometimes develops without hypertension while hypertension can be induced by ammonium salts without accompanying pulmonary œdema. We have not been able to confirm the Koenigs' observations on transection of the spinal cord because our rats invariably died soon after the operation. The outcome of all of these investigations may be summarised in the suggestion that ammonium œdema of the lungs is bound up with the sympathetic nervous control of those organs and the œdema may be labelled adrenergic nervous œdema.

During the last two years we have been using the self-same pattern of study for the investigation of other types of experimental pulmonary œdema. We have found, for instance, that the œdema which results from giving α naphthyl thiourea (ANTU) to rats cannot be controlled by drugs or devices aimed at interrupting both divisions of the autonomic nervous sytem, that the fibrin neurogenic œdema responds to block of the cholinergic nervous system and to anti-histamine drugs but not to adrenergic nerve block, though Halpern and his co-workers (Cruchaud and Vermeil, 1950) apparently were able to prevent this type of œdema by such means. We would recall that Halpern *et al.* (1950) have inhibited the pulmonary œdema caused by inhalation of chlorine and phosgene gas by the use of anti-histamine drugs, and that other workers in two World Wars have failed to obtain beneficial results by interrupting the parasympathetic and sympathetic nerve supplies to the lungs of gassed animals. This œdema would thus appear to be the result of primary endothelial damage. We would also remind the reader that hydrostatic and osmotic factors are still retained as possible causes of lung œdema although the experimental and clinical evidence for these distinctions is far from clear.

Accordingly we suggest as a tentative classification of pulmonary œdema the following scheme :—

1. Hydrostatic.
2. Osmotic.
3. Neurogenic
    - A. Adrenergic.
    - B. Cholinergic.
4. Endotheliogenic.

Future investigation may necessitate the modification of the classification but for the time being it will serve as a plan for further work.

## Summary

The intraperitoneal injection of large doses of ammonium salts induces acute pulmonary œdema in rats associated with, but independent of, intoxication of the central nervous system. Premedication of animals with sympatholytic drugs inhibits or prevents the œdema but parasympathetic blockage, whether by double vagotomy or administration of atropine, is without influence. Antihistamine drugs, too, are ineffectual. For these and other reasons it is suggested that ammonium pulmonary œdema be labelled as a type of adrenergic nervous œdema. A tentative classification of pulmonary œdemas is proposed.

We wish to thank Dr R. A. Mehrotra for preparing the diagram and photomicrographs and Mr F. J. Crew for much assistance with the experiments. Expenses were met by a grant from the Graham Research Fund.

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# CARDIAC LESIONS IN RABBITS FOLLOWING INJECTIONS OF HORSE SERUM

T. Crawford and J. R. Nassim

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(Plates CII-CIV)

Longcope, in 1915, noted myocardial lesions in rabbits which had received injections of horse serum, consisting only of round-cell infiltration with muscle-fibre degeneration. Although he found no similar lesions in his control rabbits, these changes do not differ clearly from those later described by Miller (1924) in a high proportion of apparently normal uninjected rabbits. Klinge (1929-30) described arterial lesions resembling polyarteritis nodosa in rabbits which had been given repeated horse-serum injections, and Vaubel (1932) found similar vascular lesions together with myocardial lesions resembling Aschoff bodies. Metz (1931-32) had already obtained this type of lesion following injections of bovine serum.

Since these earlier studies horse-serum injections into rabbits have been widely employed in the investigation of hyperergic tissue reactions with striking variation in the incidence and severity of lesions, and the interpretation of the results has varied correspondingly. In the present study an unusually large number of rabbits has been given horse-serum injections and the morphology of the lesions has been carefully studied and compared with that of lesions occurring in untreated rabbits and in diseases of man.

## Experimental methods

Ninety young adult male rabbits from mixed stock and weighing 1·5-2·5 kg. were given injections of horse serum. This serum was kindly provided by Burroughs Wellcome and Company and it showed an average protein content of 5·6 g. per 100 ml. (albumen 1·96 g., $\alpha$ and $\beta$ globulins 2·24 g., $\gamma$ globulins 1·40 g.). Serum dosage followed the scheme of Rich and Gregory (1943*a*), intravenous injections of 10 ml. per kg. body weight being given on the first and nineteenth days of the experiment, with a small injection of 1 ml. between the two large doses, on the seventeenth day. The animals were killed from 4 to 8 days after the last dose by means of an injection of hexabarbitone sodium and autopsy was performed immediately. The heart and other tissues were fixed in aqueous mercuric chloride followed by formol-saline. After a few hours' fixation, when firm enough to cut, the heart was sectioned transversely to give 6 to 8 thin blocks. These were embedded in paraffin and sections were cut and stained by the usual techniques, special attention being paid to the demonstration of fibrin in the lesions. To this end Weigert's fibrin stain, Mallory's phosphotungstic acid-hæmatoxylin, the picro-Mallory method (Lendrum and McFarlane, 1940) and the acid picro-Mallory modification (Lendrum, 1949) were employed.

The control material consisted of 30 rabbits from the same stock as the experimental animals. They were not given any injections. Of these controls, 15 were apparently healthy rabbits while the remaining 15 had developed some illness and either died or were killed in the same way as the injected rabbits. The illnesses were not always diagnosed but included respiratory and alimentary infections as well as certain parasitic diseases. These two groups of control rabbits are referred to in the following sections as "normal controls" and "sick controls" respectively.

## RESULTS

Lesions were found in the myocardium, valves and coronary arteries in a high proportion of the animals. In the following paragraphs a description is given of the various types of lesion encountered.

### A. *Myocardial lesions*

1. *Œdema.* Slight degrees of œdema are difficult to assess and so this condition was recorded only when there was wide separation of the muscle fibres. This was seen in 19 (21 per cent.) of the experimental animals and was not seen in the controls. It was most evident in the papillary muscles of both ventricles and in the lateral wall of the right ventricle.

2. *Lymphocytic foci.* Collections of lymphocytes were found in the myocardium of 41 (46 per cent.) of the injected and 6 (20 per cent.) of the control animals. They varied in size from small groups of a dozen or so cells up to large aggregations measuring as much as a mm. in diameter. The large lesions, however, were not numerous.

3. *Histiocytic foci.* This term is used for convenience to designate cellular collections consisting of rather indeterminate forms with the general features of tissue histiocytes (fig. 1). These cells varied from 12 $\mu$ to 18 $\mu$ diameter; they were round, oval or irregular in outline with a round or indented nucleus having a well-defined nuclear membrane. Occasional larger forms with two or more nuclei were seen and there was a varying admixture of lymphocytes and plasma cells in the lesions (fig. 2). Frequently, near the centre of these foci, some rather indistinct eosin-staining material was present. Usually it gave the staining reactions of collagen, but in 8 experimental animals lesions occurred which contained material giving the reactions of fibrin (fig. 3). In some of the older lesions delicate new-formed collagen fibres were present among the cells. These lesions occurred with no apparent relationship to blood-vessels in 15 experimental animals and one control, and in a paravascular position in 39 experimental animals (fig. 1). Similar lesions occurred as subendocardial

EXPERIMENTAL CARDIAC LESIONS

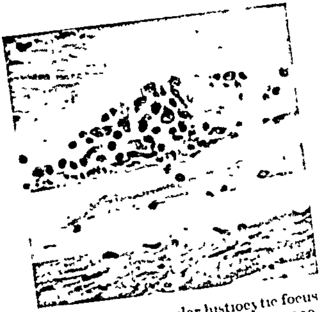

FIG. 1.—Paravascular histiocytic focus in the myocardium. Rabbit 159. Hæmatoxylin and eosin. × 200

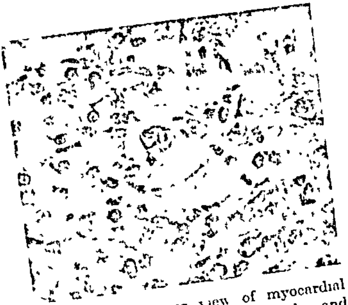

FIG. 2.—High power view of myocardial histiocytic focus showing irregular and binucleate cells. Rabbit 135. Mallory's phosphotungstic acid-hæmatoxylin. × 400.

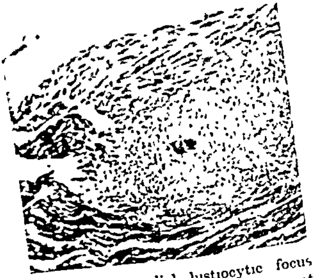

FIG. 3.—Myocardial histiocytic focus showing fibrin-like material amongst the cells. Rabbit 66. Mallory's phosphotungstic acid-hæmatoxylin. ×75.

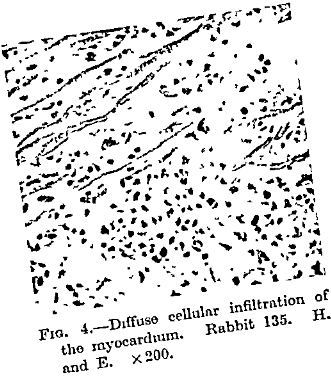

FIG. 4.—Diffuse cellular infiltration of the myocardium. Rabbit 135. H. and E. ×200.

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EXPERIMENTAL CARDIAC LESIONS

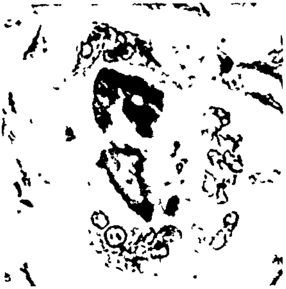

FIG. 5.—Giant cell containing fragments of necrotic heart-muscle fibre. Rabbit 42. Hæmalum-phloxine-tartrazine. ×400.

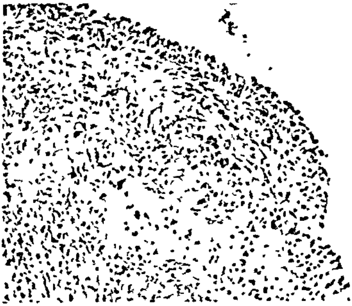

FIG. 6—Histiocytic focus in the mitral valve showing fibrin like material amongst the cells. Rabbit 26 Mallory's phosphotungstic acid-hæmatoxylin. ×100.

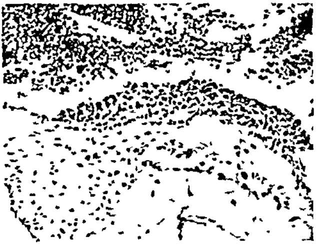

FIG 7—Endothelial proliferation forming a plaque on the surface of the mitral valve. Rabbit 47. H. and E. ×90.

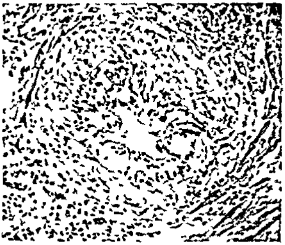

FIG. 8—Endothelial proliferation in a small coronary artery. Blood spaces have formed among the proliferated cells Rabbit 57. H. and E. ×75

foci (14 injected animals), and as subepicardial foci (14 injected animals) Altogether 49 experimental animals exhibited one or more of these varieties of the lesion

4 *Diffuse cellular infiltration* Diffuse infiltration of the myocardium by lymphocytes, plasma cells and larger mononuclear cells of the histiocyte class was seen in 32 (36 per cent ) of the injected animals and did not occur in the controls (fig 4) The area involved varied from as little as a low power microscopic field up to large tracts of the myocardium as much as a cm long The right auricle and ventricle were more frequently involved than the left

5 *Eosinophilic infiltration* Small areas of myocardial infiltration by eosinophils were seen in 6 injected animals and in one animal of the sick control group

6 *Muscle necrosis* Necrosis of muscle fibres occurred in only three of the injected animals In each case the necrosis was limited to the lateral wall of the right ventricle Individual fibres and small groups of fibres were affected and there was no apparent relationship to vascular lesions Recently affected fibres stained intensely with phloxine and showed the other staining properties of fibrin , in older lesions the staining was less intense Soon the necrotic fibres fragmented and numerous histiocytes and giant cells of foreign body type accumulated in the vicinity In many of the giant cells fragments of the necrotic muscle, still showing the staining reactions of fibrin, could be seen (fig 5) Removal of the necrotic fibres was accompanied by some overgrowth of fibrous tissue

7 *Anitschkow cells* The curious cells described by Anitschkow (1913) and since given a confusing variety of names were seen in the myocardium of 18 (20 per cent ) of the injected rabbits and 3 (10 per cent ) of the controls These cells are usually oval or elongated in shape and have an oval nucleus in which the chromatin is collected into a dense central longitudinal bar from which delicate transverse strands pass out to the conspicuous nuclear membrane Sometimes these cells entered into the formation of the histiocytic foci but frequently they lay between the muscle fibres without any clear relationship to other lesions

### B *Valvular lesions*

1 *Œdema* As with the myocardium, œdema of the valves proved difficult to assess in stained sections Definite separation of the fibres of the interstitial tissue of the cusps was, however, noted in 19 (21 per cent ) injected rabbits and 3 (10 per cent ) sick controls

2 *Histiocytic foci* Cellular foci, similar to those occurring in the myocardium, were present in the valves of 36 (40 per cent ) injected animals and of 4 (13 per cent ) controls The mitral valve was involved in each of these cases and in some the aortic or tricuspid valve was also affected As with the myocardial foci, some of the lesions con-

tained material giving the staining reactions of fibrin (fig. 6). This was seen in 6 injected and two control animals.

3. *Endothelial proliferation.* Proliferation of the surface endothelium of the valve cusp, forming a small elevated plaque, occurred on the mitral valves of 15 injected animals and was seen also on the aortic valves of some of the same rabbits (fig. 7). It did not occur in the controls.

4. *Anitschkow cells.* These cells were seen in the valve cusps of 32 (36 per cent.) injected rabbits and 3 (10 per cent.) controls.

### C. *Lesions of the coronary arteries*

1. *Nuclear changes.* The most frequent alteration in the vessels consisted in the appearance of curious nuclei amongst the muscle fibres of the media, and sometimes extending into the intima and adventitia as well. These nuclei closely resembled the nuclei of the Anitschkow cells mentioned above as occurring in the valves and myocardium, but distinct cell outlines could not be recognised and the identity of these cells with the myocardial and valvular Anitschkow cells is not certain. The change was seen principally in medium-sized and small branches of the coronary arteries and was most frequent in the arterioles of the papillary muscles of the left ventricle. It was present in 65 (72 per cent.) of the injected animals and in 8 (27 per cent.) controls.

2. *Endothelial proliferation.* Proliferation of the endothelium of the coronary arteries, forming a layer several cells thick, was encountered in 27 (30 per cent.) of the injected animals. This change usually involved the complete circumference of the affected segment but was sometimes limited to a part of it. In some cases the proliferated cells showed palisading or arch formation, and blood-containing spaces developed between the endothelial cells (fig. 8).

3. *Medial swelling.* Swelling of the medial coat of small vessels with blurring of the outlines of the muscle fibres (fig. 9) was noted in 49 injected rabbits and in one sick control. This change was often associated with the nuclear changes described above, and there were commonly cellular foci in relation to the affected vessels. The swelling and distortion of the media appeared to represent the earliest stage or mildest degree of the more severe necrotising lesions described below.

4. *Medial necrosis.* This fully developed lesion was seen in 16 (18 per cent.) of the injected rabbits. When present it usually involved the whole circumference of the affected segment of vessel, but the length of these segments varied from as little as approximately 60 μ to as much as a centimetre or more. Material giving the staining reactions of fibrin appeared in early lesions as a delicate network between the muscle fibres of the media, while in more advanced lesions the whole media was converted to an amorphous mass of the same

EXPERIMENTAL CARDIAC LESIONS

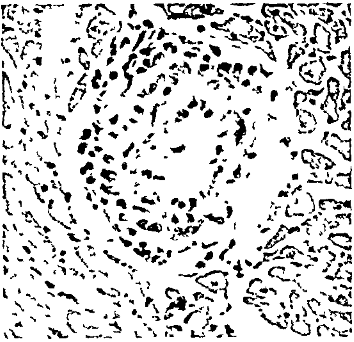

FIG 9—Medial swelling with blurring of outlines of muscle fibres in a small coronary artery There is some perivascular cellular infiltration Rabbit 159 H and E ×200

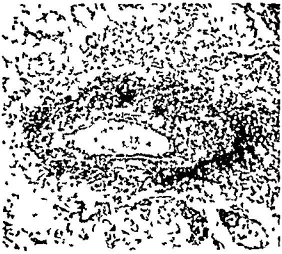

FIG 10—Necrosis in the wall of a small coronary artery Rabbit 55 Picro Mallory ×40

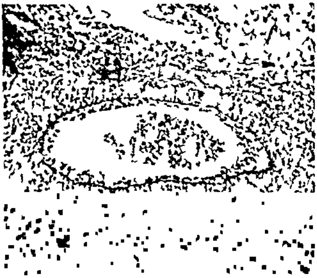

FIG 11—Stretching of the internal elastic lamina in a coronary artery with necrosis of its media Rabbit 57 Weigert van Gieson ×80

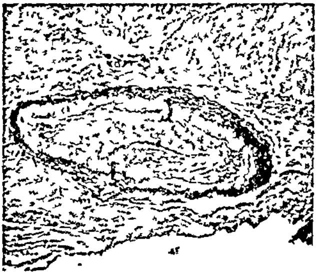

FIG 12—Scarring of a coronary artery There is fibrous thickening of the intima and adventitia, and fibrous replacement of medial muscle Rabbit 37 Weigert-van Gieson ×45

material (fig. 10). In the severest lesions there was some extension of necrosis into the intima and adventitia. Concurrently with this necrosis there was always some alteration in the internal elastic lamina. In the less severely affected arterial segments the lamina had merely lost its usual wavy form and appeared stretched and taut (fig. 11); but with more severe lesions gaps appeared in the membrane and in some instances it was totally disorganised.

5. *Arterial scarring.* In a number of the hearts arteries were seen in which there was irregular fibrous replacement of the muscular coat with defects in the internal elastic lamina and fibrous thickening of the intima (fig. 12). These were the late results of necrosis, brought about by organisation and absorption of the necrotic tissue; they were seen in 19 (21 per cent.) of the injected rabbits, seven of which also showed the active necrotic stage in nearby vessels.

## DISCUSSION

In the accompanying table the incidence of the various lesions in the injected and control groups of animals is summarised. Amongst the myocardial lesions

TABLE

*Incidence of individual lesions in injected and control rabbits*

| | Injected rabbits (90) | | Control rabbits | | |
|---|---|---|---|---|---|
| | Number | Per cent. | Normal (15) Number | Sick (15) Number | Total (30) Per cent. |
| MYOCARDIAL LESIONS | | | | | |
| Œdema | 19 | 21 | 0 | 0 | 0 |
| Lymphocytic foci | 41 | 46 | 4 | 2 | 20 |
| Histiocytic foci | 49 | 54 | 0 | 1 | 3 |
| Diffuse infiltration | 32 | 36 | 0 | 0 | 0 |
| Eosinophilic infiltration | 6 | 7 | 0 | 1 | 3 |
| Muscle necrosis | 3 | 3 | 0 | 0 | 0 |
| Anitschkow cells | 18 | 20 | 2 | 1 | 10 |
| VALVULAR LESIONS | | | | | |
| Œdema | 19 | 21 | 0 | 3 | 10 |
| Histiocytic foci | 36 | 40 | 2 | 2 | 13 |
| Endothelial proliferation | 15 | 17 | 0 | 0 | 0 |
| Anitschkow cells | 32 | 36 | 1 | 1 | 7 |
| ARTERIAL LESIONS | | | | | |
| Nuclear changes | 65 | 72 | 1 | 7 | 27 |
| Endothelial proliferation | 27 | 30 | 0 | 0 | 0 |
| Medial swelling | 49 | 54 | 0 | 1 | 3 |
| Necrosis | 16 | 18 | 0 | 0 | 0 |
| Scarring | 19 | 21 | 0 | 0 | 0 |

The figures in parentheses indicate the number of animals in each group

it is clear that neither the lymphocytic foci nor the presence of Anitschkow cells could be regarded as specific results of the injections; but even so these changes were twice as frequent in the injected as in the control animals. Evidently these are non-specific reactions which may result from a variety of stimuli, including injections of this type. This clearly applies also to the nuclear changes amongst the arterial lesions and perhaps to the œdema, histiocytic foci and Anitschkow-cell proliferation among the valvular lesions.

The lymphocytic foci in the myocardium correspond to the lesions described by Miller as " spontaneous interstitial myocarditis " and seen by him in 20 out of 34 apparently healthy adult rabbits. He found in addition cellular foci containing macrophages, but in the present series of control animals such foci were encountered in only one of the 30 rabbits whereas 54 per cent. of the injected rabbits showed this type of lesion. There can be no doubt that in most of these animals the histiocytic foci were the direct result of the injections. This applies especially to the lesions containing material giving the staining reactions of fibrin, for such material never occurred in the lesions of the control rabbits. The myocardial lesions described as diffuse cellular infiltration, eosinophilic infiltration and muscle necrosis were also almost confined to the injected rabbits and appear to be direct if less frequent effects of the injections.

Amongst the valvular lesions endothelial proliferation was encountered frequently in the injected animals and not in the controls, but the position with histiocytic foci in this situation was less clear. Our findings are in agreement with More and McLean (1949) who found " granulomatous lesions " in the valve cusps of 2 out of 25 control and 6 out of 77 injected rabbits. Their finding that lesions in the valve-ring occurred only in the injected rabbits was not fully confirmed in our material for although ring lesions occurred in 8 injected rabbits there was one example amongst the sick controls. In addition deposits of fibrin were observed in the lesions of two of the four control rabbits showing histiocytic foci in the valves. In these controls, however, the fibrin was in a more superficial position than in the injected rabbits and may well have represented a surface deposit which had become incorporated in the tissues.

With the exception of the nuclear changes, the arterial lesions are all clearly related to the injections ; and it is seen that 28 (31 per cent.) of the injected animals showed either medial necrosis or the scarring which succeeds it, or a combination of these lesions. That is to say that under the conditions of these tests approximately one third of the injected animals showed the fully developed arterial lesion. A further 24 rabbits showed only the medial swelling regarded as the early or mild lesion, and thus 52 (58 per cent.) altogether showed some degree of the vascular lesion. This incidence is lower than some other workers have observed, for Rich and Gregory (1943*a*) found arterial lesions in 70 per cent. of their animals, McKeown (1947) in 88 per cent., and More and McLean in 73 per cent. On the other hand, Alston, Cheng and Short (1947) found no lesion in 32 injected animals, and Smith and Zeek (1947), with 8 rabbits, had similar negative results. These variations in the incidence of the arterial lesions are difficult to explain and Rich, in a letter quoted by Alston, Cheng and Short, has suggested individual differences in the rabbits rather than differences in the serum as the likely explanation. In the present investigation the animals showing lesions have been fairly uniformly distributed through the series and there has been no significant difference in the incidence of lesions obtained with the different batches of horse serum. The failure of Smith and Zeek to record an example of arterial necrosis in their small group of only 8 animals is not particularly surprising for we found this lesion in only 18 per cent. of our animals ; but it is strange that Alston, Cheng and Short found no such lesion in their larger series of 32 rabbits.

The chief interest of these experimental lesions lies in the resemblance they show to the lesions occurring in certain diseases of man. In this connection the necrotic arterial lesion shows such a striking resemblance to lesions of polyarteritis nodosa in man as to suggest at once a similar pathogenesis for the two conditions, and in fact, since the revival of interest in the subject which followed Rich and Gregory's work (1943*a*), it has been widely accepted that polyarteritis nodosa results from acquired hypersensitivity to some protein or protein conjugation product It should be remembered, however, that closely similar lesions were seen by Wilson and Byrom (1941) in rats rendered hypertensive by the application of a clip to one renal artery, while Friedman, Jarman and Klemperer (1941) produced similar but less extensive lesions in rats, using a cellophane strip enveloping one kidney to produce the hypertension Smith, Zeek and McGuire (1944), employing the silk perinephritis technique to render their animals hypertensive, were able to reproduce "peri arteritis nodosa" in both rats and dogs, while Selye and Pentz (1943) found similar lesions in rats which had developed hypertension following unilateral nephrectomy and administration of desoxycorticosterone acetate and sodium chloride We are faced, therefore, with two experimental procedures each of which in suitable circum stances leads to lesions closely resembling those of human polyarteritis nodosa In the first the animals are rendered hypersensitive to foreign protein, while in the second a sharp elevation of blood pressure is induced by some suitable manœuvre It would seem that the first type of experiment leads to a tissue antigen-antibody reaction characterised by necrosis and altered staining reactions in the vessel wall, with a cellular reaction in and around the injured area, while in the second group of experiments a sudden elevation of the blood-pressure damages the inadequately adapted arteries, leading again to necrosis and altered staining reactions in the vessel wall with a secondary cellular reaction as in the former type of lesion Collateral evidence from clinical sources strongly supports the hypersensitivity experiments as representing the closer parallel to the pathogenesis of human polyarteritis nodosa, hypertension occurs in only 64 per cent of cases of polyarteritis nodosa and may come on late in the disease (Harris, Lynch and O'Hare, 1939), and many cases have now been recorded in which there was direct evidence of some form of hypersensitivity such as serum sickness (Clark and Kaplan, 1937, Rich, 1942)

Comparison of the myocardial lesions with the lesions of human rheumatic carditis shows points of close resemblance, but here again there has been much difference of opinion Vaubel noted the resemblance of the cellular nodes in the hearts of his experimental rabbits to the Aschoff bodies of rheumatism, and the close relationship of the experimental and natural lesions has been supported by Rich and Gregory (1943*b*), Hopps and Wissler (1946), McKeown (1947), More, Waugh and Kobernick (1949), Roberts, Crockett and Laipply (1949) and Wissler, Smull and Lesh (1949) On the other hand Fox and Jones (1944) regarded the lesions as only vaguely resembling

Aschoff bodies while Ehrich, Seifter and Forman (1949) consider the heart lesions not typical of rheumatic fever, but corresponding more to those of rheumatoid arthritis. Similarly, More and McLean look upon the experimental "rheumatism-like lesions" of valves and muscle as unconvincing because comparable lesions were seen in their control rabbits and the difference in incidence was not statistically significant.

The present investigation supports the first group of workers who stress the close resemblance of the experimental and rheumatic lesions. The argument hinges around whether or not the experimental histiocytic foci are in fact the counterpart of the Aschoff bodies of rheumatism. Gross and Ehrlich (1934*a* and *b*) have subdivided myocardial Aschoff bodies into two main varieties which they term the reticular and coronal types respectively, and Lendrum (1941) has confirmed this sub-division and has described the staining reactions in detail. In the reticular type there is amongst the cells a varying amount of fibrin-like material forming an interlacing network; while in the coronal type the cellular collections surround some swollen collagen fibres which do not give the staining reactions of fibrin. The descriptions we have given above of the histiocytic foci in the myocardium of the injected rabbits fit remarkably closely to these two types of lesion. Fibrin-like material was present in some of the foci in 8 animals, and these lesions correspond in general form to the reticular type (fig. 3) while in 41 animals this was not observed and all the lesions corresponded to the coronal type. The cellular constituents differed little from those seen in human lesions, though fewer large and multi-nucleated forms occurred. The differences observed are not beyond what would be anticipated in such widely differing animal species.

Of the other myocardial changes found in the injected rabbits, œdema and diffuse cellular infiltration are similar to changes commonly observed in the hearts of children dying in the early acute stage of a rheumatic attack. The extensive necrosis of muscle fibres seen in three of the rabbits, and noted previously by More and McLean, has not any clear parallel in human disease; it would seem to be the counterpart in the heart of the more frequent necrosis of muscle occurring in the coronary arteries. In the valves of the rabbit hearts nothing closely resembling the vegetations of rheumatism has been seen, though œdema and cellular foci are represented in both conditions. On the whole, the resemblance between the experimental myocardial and valvular lesions and the lesions of rheumatism, though far less striking than the similarity of the experimental arterial lesions to polyarteritis nodosa, is nevertheless sufficiently close to suggest a parallel pathogenesis, and so to lend support to the hypothesis that rheumatic lesions result from sensitisation of the tissues to some foreign antigen.

In these experiments the antigen employed has been a very complex mixture and it is desirable to determine whether particular components of the mixture are responsible for the different types of lesion observed, or whether the effects are non-specific in character and independent of the nature of the antigen. The difficulty of obtaining suitable pure antigens is considerable but if this can be achieved it will be of interest to compare the incidence of the lesions they induce with the incidence of similar lesions observed in the present study.

## Summary

Ninety rabbits were given repeated intravenous injections of horse serum and were killed 3 to 8 days after the last injection. The microscopic lesions occurring in the myocardium, heart valves and coronary arteries were compared with material from 30 control rabbits.

Significant arterial lesions occurred in 58 per cent. of the animals, and showed a striking resemblance to the lesions of polyarteritis nodosa in man. The myocardial and valvular changes, which were seen respectively in 54 and 40 per cent. of the animals, are, however, less obviously the counterparts of rheumatism. Nevertheless, the histiocytic foci show variations corresponding closely to the reticular and coronal types of Aschoff body, and the resemblance is sufficient to lend support to the view that the lesions of rheumatism are the result of a tissue antigen-antibody reaction. It is not yet clear whether the multiplicity of lesions obtained in these experiments is the outcome of the use of horse serum—a complex mixture of antigens—or whether the effects are independent of the nature of the antigen. The elucidation of this problem will require the use of suitable pure antigens.

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# HYPERSENSITIVITY REACTIONS TO HORSE SERUM IN THE BLOOD VESSELS OF RABBITS

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(Plates CV and CVI)

This paper describes experiments in which hypersensitivity reactions were evoked in animals by injecting the "shocking" dose of antigen directly into the walls of large blood vessels

## Methods

Twelve male albino rabbits, 1 8 2 1 kg body weight, were sensitised against filtered sterile normal horse serum containing no preservative by the method of Rich and Gregory (1943) Nine animals received an initial intravenous injection of 20 ml serum per kg body weight, three 10 ml per kg The subsequent dosage was 10 ml per kg for all animals except two which were given 20 ml per kg as the second dose The latter was administered on the nineteenth day, the rabbits being partially desensitised by an intravenous injection of 1 ml serum given 24 hours previously In experiments with multiple injections the time spacing was 12 15 days, without recourse to previous desensitisation One rabbit died immediately after the first desensitising dose and another 24 hours after the fourth massive dose

An intradermal injection of 0 2 ml horse serum was first given on the twelfth day for skin testing The ensuing reaction was pronounced in a number of rabbits but consisted of a faint erythema, 5 7 mm diameter, in others Later tests were much more active in their responses, an erythematous swelling 3 3 5 cm diameter, with hæmorrhage and even necrosis developing in 5 rabbits 24 48 hours after the test injection Two rabbits, however, remained completely refractory to skin testing even after seven injections and these were excluded from the experiment A transient rise in temperature up to 103 5° F was observed in all animals between the sixth and ninth days after the first injection A well marked cutaneous flush was noted in the ears of two rabbits on the fifth and sixth days , this faded after 72 hours

The final "shocking" arterial injections were made 8 10 days after the last intravenous dose of horse serum A skin test performed 48 hours before this operation proved to be strongly positive in all of the animals Under Nembutal and ether anæsthesia and with strict asepsis the femoral arteries were exposed and separated from the accompanying structures and 0 1 ml horse serum was injected into the adventitia of well separated, small segments of the vessels A common carotid artery was similarly infiltrated in each rabbit The animals were killed at varying time intervals over a period of 3 45 days

Three control male rabbits from the same stock and of similar weight to the experimental animals were given 10 ml per kg normal saline intravenously, the dose being repeated after 19 days. Ten days after the second injection a common carotid and both femoral arteries in each animal were infiltrated with normal horse serum as in the case of the experimental animals. The controls were kept under similar conditions to the latter and were killed at various time intervals.

Injected segments of arteries from all animals were dissected out carefully with the surrounding muscle, fixed in 10 per cent. formol saline, cut at six different levels by the paraffin technique and stained with Ehrlich's acid hæmatoxylin and eosin, van Gieson and Weigert's elastic stain. In addition, random pieces of heart, lung, kidney, liver, spleen, ascending aorta, and in some cases mesenteric artery were sectioned as a routine procedure.

## RESULTS

1. *Acute reactions.* Arterial lesions varied greatly from animal to animal and with the time interval elapsing after the "shocking" injection.

A. A mild response, encountered in some vessels 5-7 days after local infiltration, merely consisted of slight œdema and lymphocytic and large mononuclear cell infiltration in the perivascular tissue and the adventitial coat. The endothelium of the vasa vasorum was invariably swollen and prominent and the vasal walls appeared œdematous and infiltrated with mononuclear cells. No alteration could be detected within the media and intima of the main vessel.

B. In the next grade of reaction both the adventitia and media were œdematous and infiltrated with lymphocytes while pyknotic change in the nuclei of some of the muscle cells of the vessel wall denoted damage to these elements.

C. The first evidence of really serious disturbance consisted of a well-marked inflammatory reaction in the perivascular tissue and the adventitia, the exudate containing numerous histiocytes, lymphocytes, large plasma cells, and eosinophils. In one instance a multinucleated giant-cell was discovered. The vasa vasorum were also involved in this response. The media and intima seemed œdematous and more cellular than usual, the endothelial lining cells were frequently swollen and desquamating but the internal elastic membrane appeared to be intact.

D. A truly severe type of damage was found in most rabbits, as shown by marked œdema and massive cell infiltration of the media and adventitia, damage and necrosis of fibrous tissue and muscle and thinning, straightening and fraying of elastic fibres. Polymorphonuclear leucocytes and eosinophils, too, were numerous in these coats. The intima was full of inflammatory cells, subendothelial œdema was striking and endothelium had proliferated to form minute papillary processes and ingrowths. The internal elastic lamina remained intact though sometimes it seemed less wavy. Collagenous fibres were often well separated by œdema, swollen and hyalinised, especially in the intima. Apparently the medial lesions could commence in any part of the muscular layer for in some segments it involved the inner third

HYPERSENSITIVITY

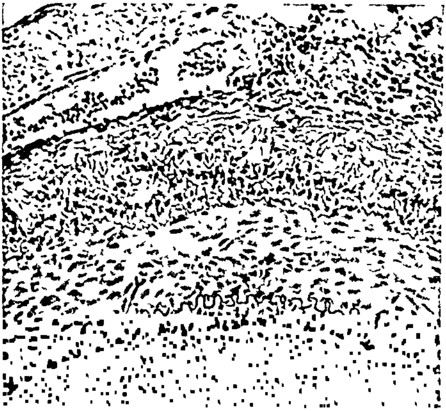

FIG. 1.—Femoral artery of sensitised rabbit 5 days after injection of serum into adventitia. Perivascular cellular exudation, slight endothelial proliferation with palisading of nuclei and subendothelial œdema. A few pyknotic nuclei in the media. Hæmatoxylin and eosin. × 170.

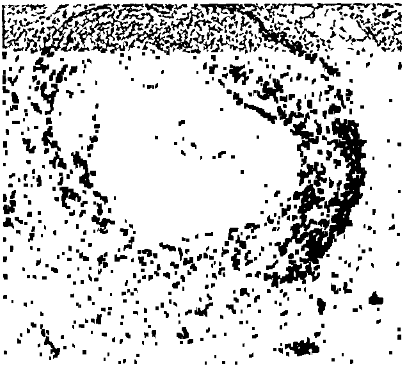

FIG. 2.—Common carotid artery of sensitised rabbit 6 days after injection of serum into adventitia. Extensive necrosis and œdema of media, bands of fibrinoid in adventitia and media, proliferating endothelium and thrombosis. All coats show a heavy cellular exudation. H. and E. × 80.

of that coat only, while in others the outer third alone was necrotic and inflamed.

Every now and then endothelial proliferation produced irregular thickening of the intima and masses of these cells together with various inflammatory cells could be traced into recently formed thrombi occluding the lumen. Occasionally such cells were aggregated to form follicles not unlike those described by Buerger (1908; 1914-15) in cases of thromboangiitis obliterans. In such instances fibrinoid was particularly well developed and much destruction of elastic fibres had occurred in the media.

E. In one animal the arterial wall response was highly reminiscent of an Arthus reaction in the skin. Fibrinoid degeneration was now well developed, eosinophilic leucocytes were numerous and muscle destruction led to disorganisation of the wall.

2. *Chronic reactions.* Animals allowed to survive for 45 days showed chronic or healing arteritis at the sites of sensitisation. Localised medial damage underwent repair by the proliferation of fibrous tissue cells. The intima became irregularly thickened with cellular connective tissue and new elastic fibres contributed greatly to localised intimal thickening. Often the internal elastic lamina appeared to be disrupted and fragmented, leaving strips of membrane with wavy contours. No cellular activity could be associated with this change. The adventitia was also thickened in places because of fibrosis. In this way quite pronounced localised arteriosclerosis could be induced which led to diminution in size of the lumen and was associated, from time to time, with organising thrombosis.

3. *Control reactions.* In the control animals no significant changes were found either in the intima or in the media, but a slight or moderate degree of leucocytic infiltration was constant in the adventitia and perivascular tissues during the first few days after injection of horse serum. Later, mononuclear cells and fibroblasts appeared and gave place to slight fibrosis within 15 days. This reaction was obviously identical with the first type of response met with in sensitised animals.

Sections from heart, lung, kidney, liver, spleen, ascending aorta and mesenteric artery gave no evidence of arteritis despite the fact that the animals were sensitised under conditions which have led to arterial lesions in such sites (Rich and Gregory, 1943; More and McLean, 1949).

## DISCUSSION

These experiments show that arteries can be sensitised to a foreign protein and that a series of acute reactions can be so induced which pass on to thickening of the affected arterial segment from fibrosis and elastosis. Such changes resemble very closely those which have been established in conditions of hypersensitivity in man and by less certain experimental methods in animals.

A critical survey of the already vast literature on hypersensitivity (see, for instance, the valuable reviews by Klinge, 1929-30, 1930; Rössle, 1932, 1933; Apitz, 1933; Pagel, 1939; Rich, 1942; Teilum, 1945-46 and Bergstrand, 1946, 1950), leads to the rather astonishing conclusion that acceptable observations on blood-vessel changes in sensitised human beings are far from common. The most satisfactory of these are worthy of consideration. Clark and Kaplan (1937) recorded interesting arterial lesions in two patients dying from serum sickness shortly after the injection of horse serum. These included fibrinoid swelling of the wall, sometimes frank necrosis, infiltration of some or all of the coats with mononuclear cells and eosinophilic leucocytes, proliferation of the lining endothelium and the development of perivascular granulomata. The association of periarteritis nodosa with hypersensitivity has often been claimed. Gruber (1925) may have been the first pathologist to suggest this relationship; at any rate many others have followed suit, being influenced especially by the frequency of eosinophilic infiltration in the vascular lesions, a finding which is generally thought to be essential to hypersensitivity reactions. A number of workers, too, have reported cases of periarteritis nodosa associated with bronchial asthma. Kallós and Kallós-Deffner (1937) recorded 29 cases of asthma in which death took place during an attack. Histological studies showed definite periarteritis nodosa in one and lesions suggestive of periarteritis in another. Rackemann and Greene (1939) reported 8 cases in which asthma and periarteritis nodosa were coincidental. Among 229 instances of periarteritis collected from the literature were 19 in which the patient had suffered from asthma. Even more direct evidence is available. Eason and Carpenter (1937), for instance, described periarteritis in a case associated with serum sickness. The careful studies of Rich have aroused much interest in the correlation. Rich recorded 8 cases of hypersensitivity, shown by serum rashes, after the therapeutic injection of foreign serum, with or without sulphonamides. Recent periarteritis was found in all. The evidence for a close association was especially good in one instance, where tissue was examined before and after the development of hypersensitivity. Arterial lesions included fibrinoid necrosis of the media and perivascular infiltration with polymorphs and monocytes. Eosinophils were present in most cases. Fibroblasts were numerous in the affected arteries of a patient who lived for 3 weeks after the appearance of urticaria. Rich showed that periarteritis could develop as soon as 5 days after the onset of hypersensitive reactions.

There is no need to recapitulate the story about sulphonamides as sensitising agents for man since the investigations of Lederer and Rosenblatt (1942), Merkel and Crawford (1942), Rich (1942), Black-Schaffer (1945), French (1946), Lichtenstein and Fox (1946), and More *et al.* (1946) have established their potency in producing arterial lesions remarkably like those now well known in idiopathic periarteritis nodosa.

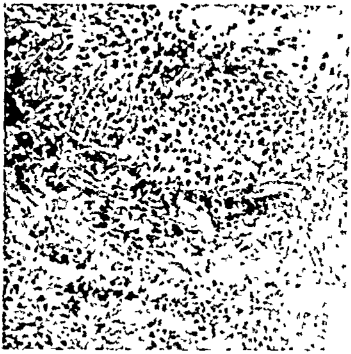

FIG. 3—High-power view of part of fig. 2. Proliferating [illegible] mixture of polymorphs and lymphocytes form a [illegible] E. ×250.

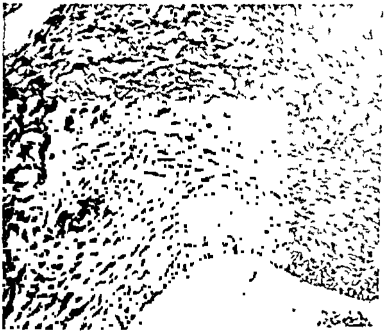

FIG 4—Femoral artery of sensitised rabbit 7 days after injection of serum into *adventitia*. Two irregular patches of fibrinoid are seen among degenerating cells in the media and adventitia Perivascular exudation and intimal proliferation H and E. ×170.

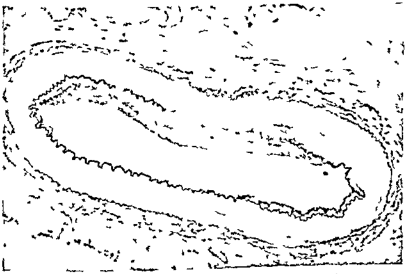

FIG. 5—Femoral artery of sensitised rabbit 45 days after injection of serum into adventitia. Elastosis of intima. Weigert's elastica and neutral red. ×65.

The position is not such a happy one with experimental hypersensitivity since workers disagree about their results and details of experiments are often scanty. However, the investigations of Klinge (1930), Vaubel (1932), Masugi (1933), Junghans (1933-34), Masugi and Sato (1934), Heinlein (1937), Rintelen (1937), Stecher (1937), and Stenn (1938) and more recently of Rich and Gregory (1943) and More and McLean (1949) cannot be passed over lightly, and there is much to be said for the conclusion reached by More and McLean that the arteritis of hypersensitivity is " basically similar to the lesions of periarteritis nodosa in man ". Experimental studies thus suggest that hypersensitivity carries with it an underlying fundamental pattern of disturbance in the blood vessels as well as in the inner coat derivatives of the heart. Damage to the endothelium seems to be a prominent feature; indeed, we may go further and agree with Bergstrand (1950) in maintaining that this is the most prominent change in anaphylactic reactions. My own observations certainly convince me of the truth of this generalisation. Associated with the endothelial disturbances are changes in connective tissue, including swelling of fibres, focal splitting of collagen into reticulum, fibrinoid degeneration, fragmentation of fibrils and proliferation of fibroblasts leading to scar formation. As the result of endothelial damage, whether it affects the endothelium of the intima or of the vasa vasorum, we encounter focal or diffuse necrosis of the vessel, secondary thrombosis, sometimes hæmorrhage and, of course, exudation of plasma (œdema) and of cells (cellular infiltration). The cellular response is mainly of the mononuclear type, but eosinophils are just about constant in their occurrence and usually numerous at one or other stage of the reaction.

I have encountered precisely these findings and for this reason I maintain that the rather artificial nature of my experiments may be tolerated. There can be no doubt about the production of hypersensitivity reactions in the arteries which I have studied. It is therefore all the more encouraging for the student of hypersensitivity that the pathological responses so induced are in close agreement with those obtained by methods which imitate the human set-up rather better than mine although they require a certain amount of faith in uncontrollable factors.

## SUMMARY

A hypersensitive state has been induced in rabbits by repeated intravenous injections of sterile horse serum. The introduction of small amounts of this antigen directly into the walls of large arteries of the sensitised animals is followed by various degrees of severity of acute arteritis, which commences in close association with the endothelium of the intima and of the vasa vasorum. Healing of these lesions leads to fibrosis and elastosis confined to the affected segments of the artery.

I am grateful to Professor G. R. Cameron, F.R.S., for his help in carrying out this investigation and to Mr F. J. Crew for much assistance with the experiments. Expenses were met with a grant from the Graham Research Fund.

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616 . 831—008 . 99 (*Ascaris*): 616 . 832 . 21—002 . 1

# AN ASCARIS LARVA IN THE BRAIN IN ASSOCIATION WITH ACUTE ANTERIOR POLIOMYELITIS

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(PLATES CVII-CXI)

SINCE 1831, when Lepelletier reported fatal convulsions in two children with alimentary ascariasis, there have been numerous reports in the continental literature of round-worm infestation accompanied by varied and severe neurological symptoms. The relationship of the symptoms to the ascariasis, however, remains obscure. The severity of cerebral ascariasis can be inferred from Fernando and Balasingham's recent (1943) report of fourteen cases suggesting encephalitis and one suggesting meningitis, in a series of 162 cases of acute ascariasis. Eight of these patients certainly died and three others were believed to have died at home.

The clinical appearances of cerebral ascariasis are those of generalised or Jacksonian convulsions (Duprey, 1903 ; Sironi, 1921) or those suggestive of an acute inflammation of the brain or meninges (Taillens, 1906 ; Hughes, 1920 ; Turcan, 1922 ; Lafforgue, 1923 ; Abel and Brenas, 1925 ; Renaud, 1929 , Schachter, 1934 ; Hautefeuille, 1935 ; Peney, 1940). Changes in the cerebro-spinal fluid suggestive of these more familiar neurological diseases have been encountered several times.

The unique features of the case reported here are that an encapsulated larva was found in the brain and that the neurological symptoms were largely due to a second disease agent, the virus of poliomyelitis.

## CLINICAL HISTORY

The patient, a girl aged 6, was admitted to the Westminster Children's Hospital, under the care of Dr N. O. Richards on 29th December 1949. She had been vaccinated when aged a few weeks and immunised against diphtheria at the age of 12 months. She had measles in March 1948 and a tonsillectomy in March 1949. She had had a dry cough in the winter since the first year of life. The patient did not mix much with other children, but was always playing with cats and dogs and was in the habit of sucking her thumb. She was an excitable child, often feverish before parties and had talked in her sleep ever since she went to school. For the last six months she had ground her teeth at night.

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* In receipt of a grant from the Institute of Neurology

Neither the patient nor any member of the family had ever been known to suffer from worms. On 24th December 1949 she complained of a sore throat. On the following day she was flushed, listless, constipated and vomited several times. On 26th December she complained of pain in the back of her neck and continued to vomit. By the evening of 28th December she was delirious, "rolling her eyes and baring her teeth like an animal". On admission she was a febrile (temperature 102° F., pulse 140 per min.), flushed, drowsy, well-nourished child showing frequent convulsive movements of the arms and legs and occasionally crying out. She had a vacant stare but could be made to answer questions. There was pallor of the mucous membranes; the mouth was dry, the tongue coated, the throat inflamed and the pharynx contained a large quantity of green mucus, some of which was vomited from time to time. The left ear drum was normal, the right was dull and pink in its upper part. There was a slight nasal discharge and some crusting around the nostrils. The respiratory and cardio-vascular systems were normal. Nothing abnormal was found on abdominal examination. The pupils reacted briskly to light and there was no nystagmus. The fundi were normal. There was neck stiffness and slight head retraction. Kernig's sign was positive. The tendon reflexes were present but diminished in the arms and brisk in the legs. The abdominal reflexes were present but the plantar responses were extensor. There was no loss of power in the limbs.

Lumbar puncture yielded a slightly turbid fluid which was not under increased pressure. Examination of the fluid showed neutrophil polymorphs 85 per c.mm.; lymphocytes 73 per c.mm.; R.B.C. 485 per c.mm.; protein 32 mg./100 ml.; Pandy test negative; Nonne Apelt test negative; chlorides 730 mg./100 ml.; sugar 50-90 mg./100 ml.; no organisms seen in Gram film; no acid- and alcohol-fast bacilli seen in Z.N. films; culture yielded no growth.

The patient was given 10,000 units of penicillin intrathecally, intramuscular penicillin and oral sulphamezathine. The convulsive movements continued. She was incontinent and during the night suddenly collapsed and died. Stools from the parents and a surviving sister were examined for ova by direct smear and concentration methods, but none was found.

## POST-MORTEM EXAMINATION

The body was that of a well-nourished female child. The pupils were central, circular and equal. There was no cyanosis. There were a few petechiæ around the neck. Several lymph nodes were palpable along the anterior border of the right sterno-mastoid. No other nodes were palpable.

Adenoid tissue around the naso-pharynx was prominent. The naso-pharynx contained a little frothy mucus. The trachea and main bronchi were slightly inflamed and contained some inhaled gastric contents. The lungs were normal.

The blood vessels over the surface of the brain and cord were somewhat congested; the meninges were otherwise normal. The cut surface of the brain showed no abnormality to the naked eye, except in the medulla where there were a number of small hæmorrhages in the floor of the fourth ventricle.

The stomach contained the sulphamezathine given before death. There were no other abnormalities and no worms were found in the

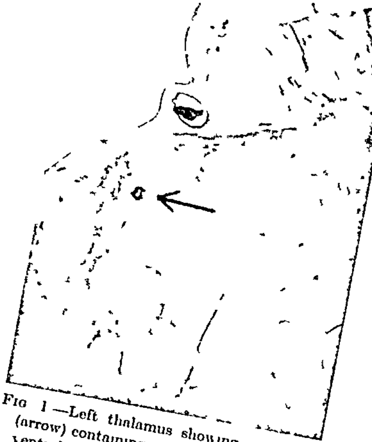

FIG 1—Left thalamus showing granuloma (arrow) containing ascaris larva in nucleus ventralis lateralis Hæmatoxylin and van Gieson ×6

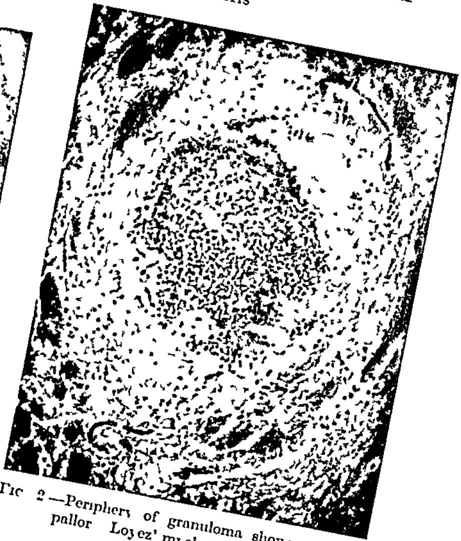

FIG 2—Periphery of granuloma showing halo of pallor Loyez' myelin stain ×140

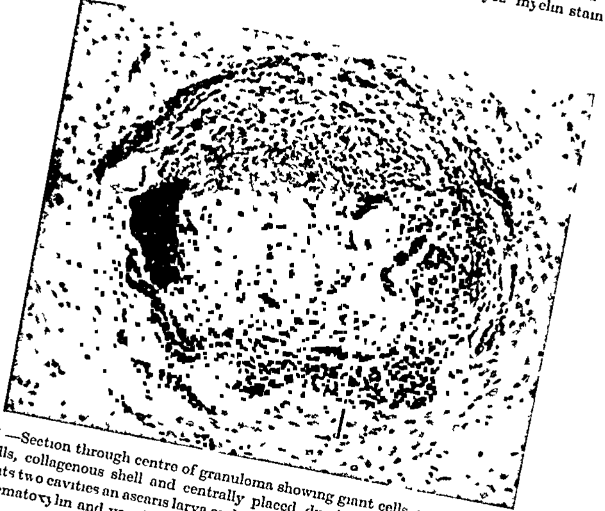

FIG 3—Section through centre of granuloma showing giant cells, zone of inflammatory cells, collagenous shell and centrally placed dumb bell shaped cavity containing in its two cavities an ascaris larva and a giant cell with a few fibroblasts, respectively Hæmatoxylin and van Gieson ×188

## CEREBRAL ASCARIASIS AND POLIOMYELITIS

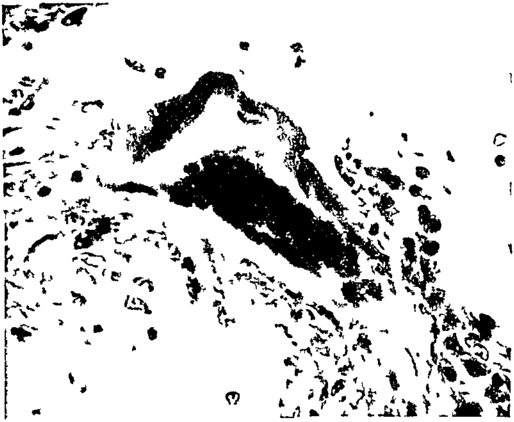

Fig 4 —Same section as fig 3 showing the largest giant cell
Hæmatoxylin and van Gieson × 605

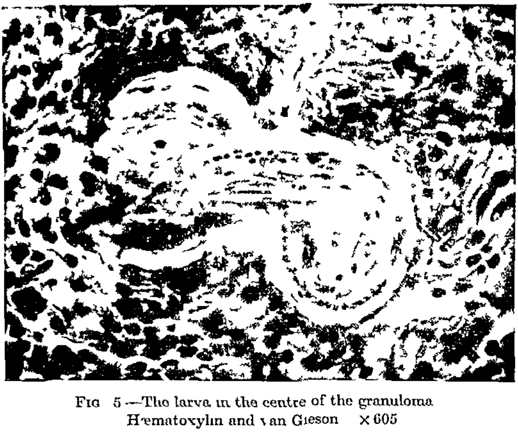

Fig 5 —The larva in the centre of the granuloma
Hæmatoxylin and van Gieson × 605

gut. Apart from slight dilatation of both ventricles, the heart was normal. The Malpighian bodies of the spleen were prominent and there were a few moderately enlarged fleshy lymph nodes at the bifurcation of the trachea and in the mesentery. The urogenital system, liver, gall-bladder, pancreas, and adrenals were normal.

## HISTOLOGY

The liver, kidneys, heart and lungs were normal. After the larva was found in the brain the small pieces of liver, kidney and lung which had been preserved were serially sectioned, but no evidence of larval passage was found.

Blocks were taken from the 1st lumbar, 8th thoracic and 4th cervical segments of the spinal cord, from the pons, from the mid-brain, left thalamus and hippocampus at the level of the optic tract, from the left occipital lobe, from the left pre- and post-central gyri and from the cerebellum. Frozen sections from each of these blocks were stained with Scharlach R and the remainder of the tissue embedded in celloidin. Celloidin sections from each block were stained with thionin, Mallory's phosphotungstic acid-hæmatoxylin, van Gieson, and Loyez' stain for myelin. Sections were also impregnated by the Gros-Bielschowsky method for axis cylinders. Thirty sections in series through the thalamus and passing from one side to the other of the granuloma reported below, were stained severally by Leishman, *Verhoeff's method for elastic tissue and Heidenhain's azan stain, as* well as by the above methods. One section was impregnated by Gordon and Sweet's technique for reticulin. In a search for larvæ, twenty-two sections through the 4th cervical segment of the spinal cord stained with thionin or Leishman, were examined.

There appear to be three quite distinct types of lesion in the nervous system. These comprise :—

(1) A granuloma situated in the nucleus ventralis lateralis of the left thalamus and containing in its centre an ascaris larva.

(2) Peri-arteriolar hæmorrhages and other "non-inflammatory" vascular changes.

(3) A polio-encephalomyelitis mostly remote from lesions (1) and (2).

### (1) *The granuloma in the nucleus ventralis lateralis of the left thalamus*

A granuloma (fig. 1) is present in this nucleus, immediately ventral to and nestling in the concavity formed by the fibres of the anterior thalamic projection and the internal medullary lamina. The granuloma measures 0·4×0·5 mm. in the coronal plane and the antero-posterior diameter is estimated from sections as 0·36 mm. It is surrounded by a zone of pallor (fig. 2) in Loyez' preparations. Centrally there is a dense shell made up of thick collagenous fibres among which are a few irregularly-shaped hyperchromatic fibroblast nuclei. This shell is surrounded by a delicate tracery of collagenous fibres in the interstices of which are fibroblasts, plasma cells and lymphocytes. Impregnation of the reticulin fails to reveal any reticulin fibres beyond the collagenous mesh. Outside this loose collagenous layer are densely packed lymphocytes and plasma cells, surrounded in turn by less frequent plasma cells and proliferating microglia and astrocytes, the latter forming only a few fresh fibres. There is also new formation of capillaries. In azan preparations the extreme periphery of the granuloma is evident macroscopically by reason of its more intense staining with the·anilin blue. The myelin sheaths in the vicinity of the granuloma appear to have been pushed aside, so producing the clear halo seen in the Loyez' preparation. A few sheaths show slight beading; their axis cylinders appear normal. The concentric zones making up the outer part of the granuloma are not quite symmetrical, the cellular zone being wider posteriorly. The dense collagenous shell encloses a dumb-bell-shaped cavity (fig. 3) containing in one chamber a giant cell with four nuclei of moderate chromatin content. The other chamber contains the larva. Medial to the parasite is an elongated giant cell (fig. 6, arrow) measuring more than 120 μ in length but only 12 μ in breadth. On the ventral border of the collagenous shell is a third, triangular giant cell (fig. 4) measuring 105×95×55 μ and containing large numbers of irregularly shaped hyperchromatic nuclei arranged in a central cluster as well as forming a peripheral border to the cell. It is probable that the last two giant cells are continuous with one another so as to surround the larva for a quarter of a circle, though separated from it to a large extent by the ingrowth of the collagenous shell which produces the isthmus of the dumb-bell.

*Description of the parasite* by Professor J. J. C. Buckley, D.Sc. of the London School of Hygiene and Tropical Medicine.

" The parasite in the brain section (figs. 5 and 6) is a larval nematode which is complete except for a small portion of the posterior extremity which has been removed in sectioning. Measured in one plane it is 295 μ long but owing to curvature in the third dimension this is an underestimate and it may be taken to be about 310 μ. The missing tail portion would make the total length about 330-350 μ long. Its breadth is 15 to 20 μ. The muscular œsophagus is 95 μ long by 7 μ in breadth, expanding to a posterior swelling of 10 μ in breadth. This type of œsophagus and the small dimensions exclude the possibility of the larva being that of *Trichinella spiralis* and all the evidence points to its being a migrating larva of *Ascaris lumbricoides*, but the possibility that it might be the larva of an ascarid from a dog or cat cannot be entirely excluded. According to the dimensions given by Ransom and Cram (1920-21) of ascaris larvæ recovered from various organs in experimental animals at varying intervals after infection, the age of the larva in the brain may be anything from 3 to 7 days old. In this abnormal habitat it is possible, however, that development may have been arrested and that the dimensions are not a true indication of the age."

(2) *The "non-inflammatory" vascular changes*

These are present most strikingly in the white matter of the gyrus occipito-temporalis lateralis, but also in the putamen, the grey matter of the uncus, the gyrus dentatus, the internal medullary lamina of the thalamus, the nuclei ventralis lateralis and antero-ventralis and the anterior thalamic projection, the tail of the caudate nucleus, the cortex of the pre- and post-central gyri and that on either side of the calcarine fissure, the cortex of the cerebellum, the pons, notably in the region of the descending root of the trigeminal nerve, and in the floor of the fourth ventricle and the cervical region of the spinal cord. However, the last two sites are so heavily infiltrated with inflammatory cells that it is not possible to exclude the polio-encephalomyelitis as the cause of the vascular lesions in these particular areas.

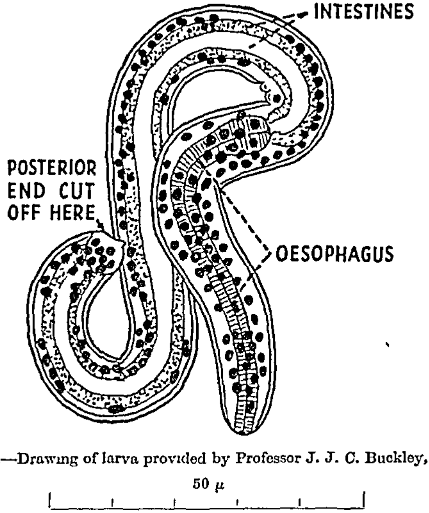

FIG. 6.—Drawing of larva provided by Professor J. J. C. Buckley, D.Sc.

The lesions mainly affect small arteries and arterioles and consist of intravascular clotting, extravasation of all or some of the constituents of the blood and secondary changes in the surrounding tissues. The thrombi are fresh and frequently occupy only the parietal part of the vessel lumen. Delicate fibrin threads frequently extend across the Virchow-Robin space into the brain parenchyma. More extensive fibrin exudate surrounds a thrombosed pial vessel in the cerebellum (fig. 7). Larger vessels often display an exudate of homogeneous material into a greatly distended extra-adventitial (His') space (fig. 8). This material stains pale pink with phosphotungstic acid-hæmatoxylin, orange with van Gieson's stain, green with Masson's stain and blue with Heidenhain's azan stain. It is often present in globular form (fig. 9) when it must be distinguished from the globules derived from the occasional degenerated myelin sheath, which, however, stain darkly in the Loyez' method (fig. 10, arrow). Gros-Bielschowsky preparations show no change in the axis cylinders near the vessels. Several vessels show striking perivascular hæmorrhages almost entirely confined to the perivascular space and following the course of the vessel for a considerable distance (fig. 11). Such vessels are frequently the site of intravascular clotting and clotting is often seen also in the extravasated blood (fig. 12). The surrounding myelin sheaths frequently show vacuolation with an occasional karyorrhectic glial nucleus. In other vessels surrounded by extravasated blood the adventitial histiocytes which are present in excess are pyknotic. Occasionally the formation of astrocytic fibres is increased near a vessel with a distended perivascular space but this is nowhere conspicuous.

### (3) *Non-suppurative polio-encephalomyelitis*

These inflammatory changes extend the whole length of the spinal cord, affect the dorsal part of the pons, the substantia nigra, the corpora mamillaria and, in a few sections only, the cerebral cortex in the depths of the fissure of Rolando. The changes are most marked in the cervical segments of the spinal cord. Here the lesions are almost confined to the grey matter which is severely affected. While about one-third of the anterior horn cells appear healthy, many show eosinophilic degeneration (fig. 13), chromatolysis (fig. 14) or cytolysis. Comparatively few cells show any but the end stages of neuronophagia although a few show early invasion by microglia, some of which lie in digestion chambers. The end stages, by contrast, are prominent and represented by numerous nodules of microglia some of which are visible with the naked eye. The posterior horns are more diffusely infiltrated with microglia and a few plasma cells. The bases of the posterior horns show perivascular hæmorrhages and, in one section, a triangular area 0·3 × 0·15 mm. of homogeneous material has resulted from the transudation of plasma into necrotic tissue. The white matter of the cord is œdematous. Almost all vessels show conspicuous lymphocytic cuffing. The anterior median fissure contains a moderately severe meningeal exudate consisting mainly of lymphocytes but also including many hydropic plasma cells and histiocytes, and an occasional eosinophil cell.

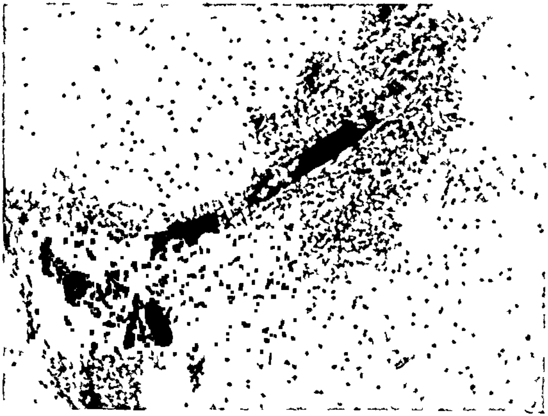

FIG 7—Cerebellum showing thrombosed pial vessel and exudate of fibrin Phosphotungstic acid hæmatoxylin ×160

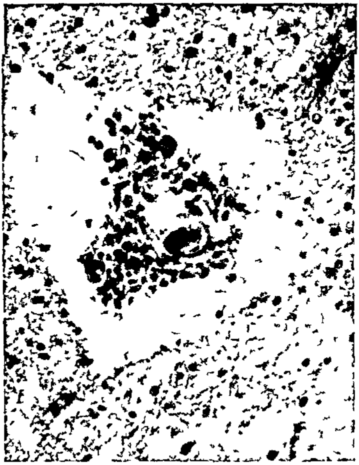

FIG 8—Section through hypothalamus showing arteriole with Virchow Robin space filled with lymphocytes His space is occupied by a pale staining vacuolated material A few astrocytic fibres can be seen in the surrounding tissue Phosphotungstic acid hæmatoxylin ×300

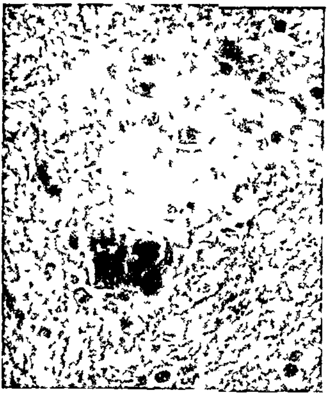

FIG 9—Same section as fig 8 showing globular bodies in His space Heidenhain's Azan stain ×560

Sections through *the pons* near the most rostral part of the ventral spinocerebellar tract, show inflammatory foci in the region of the motor and sensory trigeminal nuclei and the preposital nucleus, in the floor of the fourth ventricle close to the mid-line and scattered in the reticular formation both in the midline and further laterally, but not, however, ventral to the superior olivary nuclei. An occasional swollen cell with wrinkled nuclear membrane is seen in the superior olivary and lateral tegmental nuclei. The substantia nigra is heavily infiltrated with microglial and paravascular nodules, the nuclei of the cells making up the latter resembling those of adventitial cells rather than those of the microglia. Apart from an occasional vessel cuffed with lymphocytes and polymorphonuclear leucocytes in the globus pallidus, the basal ganglia show no other evidence of participation in the polio-encephalitis. In frozen sections stained with Scharlach R, small aggregations of nuclei—probably microglial—are seen in the cortex of the precentral gyrus. It is not possible to detect such nodules in sections from the colloidin embedded tissue.

## Discussion

### (a) *Histological appearances and diagnosis*

The presence of ascaris larvæ has not previously been reported in the brain of man or of any other animal, except following the experimental administration of the eggs of this nematode. Other or unidentified nematodes have been found accidentally in the brains of a few animals (Cahall, 1889; Bunzl, 1904; Widakowich, 1905). In 1921, Fulleborn (1921*a*) demonstrated *Ascaris lumbricoides* larvæ in the brains of experimental animals to whom large numbers of ripe ascaris eggs had been fed. He observed no clinical symptoms attributable to the migration of the larvæ through the brain, and none followed the intracarotid injection of many thousands of the energetically boring filariform strongyloides larvæ. Fulleborn (1922) further demonstrated histologically an ascaris larva in a cerebral capillary twenty-five hours after the feeding of eggs and Höppli (1923) found an ascaris larva entering one of the cerebral ventricles twenty-four hours after infection. Fulleborn (1921*b*) recovered larvæ of another nematode (strongyloides) from the lungs three minutes after injecting them into the carotid artery. The extremely rapid passage of the larvæ through the cerebral vessels may be completely passive, notwithstanding the comparatively large size (260×12-23 $\mu$) of the smallest ascaris larvæ, for Fulleborn (1922) has shown that coccidioides oöcysts 20 $\mu$ in diameter pass through the cerebral capillaries of a dog. While the majority of migrating nematode larvæ in such experiments appear to traverse the cerebral circulation uneventfully, a few escape into the brain parenchyma. This may be due to their boring activity as suggested by Fulleborn's (1921*b*) observation that striking alveolar hæmorrhages only occur after the intravenous injection of *living* strongyloides larvæ. Yokogawa (1923) saw as many as ten larvæ in the cerebral ventricles in one section and suggests that the larvæ may reach the venous circulation by active movement through the brain and into the veins of the choroid plexus.

The effects of the larvæ on the brain are limited by the paucity of reaction on the part of nervous as opposed to other tissues (Höppli, 1923, 1927; Yokogawa, 1923). Greenfield and Pritchard (1937), however, in a report on two cases of cerebral schistosomiasis, state that the necrotic tissue containing the eggs was surrounded by fibroblastic and endothelial cells, which separated it from a wall of collagenous fibres and inflammatory cells. A few multinucleated giant cells were seen in the fibroblastic layer and occasionally partially enveloped an ovum. Perivascular hæmorrhages are the most constant evidence of migrating larvæ (Yokogawa) and are characteristically confined to the perivascular (His') space. Where larvæ are halted in the brain substance, very little reaction occurs. Höppli (1923) saw no reaction around belascaris larvæ in the brains of guinea-pigs six weeks after the latter had been fed with ripe eggs but Yokogawa and Yamaguchi (1925) saw foci of softening with infiltration by a few eosinophils. Yamaguchi was unable to relate these foci to larvæ lying free in the brain tissue.

From the extensive experimental studies of Fülleborn (1927) and Höppli (1923, 1927) much is known of the more striking reactions of other tissues to the presence of larvæ. Small cellular infiltrations are present in the liver a few hours after feeding a guinea-pig with ascaris larvæ (Höppli, 1927) and irregular circular and band-like infiltrations of polymorphs and eosinophils with preservation of the reticulin net were observed by Höppli (1923) five days after infection. He similarly noted small necroses in the liver with infiltration by leucocytes and epithelioid cells eight days after feeding a guinea-pig with belascaris larvæ. At three weeks the liver was studded with connective tissue nodules resembling foreign-body tubercles and showing epithelioid cells, polymorphonuclear leucocytes and mononuclear cells with the larvæ in the centre of the nodule in a loose network of collagen fibres. Similar but thicker fibres formed a peripheral ring, outside which there was a loose connective tissue meshwork infiltrated with polymorphonuclear leucocytes. Höppli later (1927) pointed out that the infiltration may so soften a tissue as to permit of wandering of larvæ previously impossible.

From Professor Buckley's report, it can be seen that the encapsulated nematode in the thalamus of our case is a larval ascarid, in all probability *Ascaris lumbricoides*. From its size it would appear to be at least five days old; however, Fülleborn (1920) states that strongyloides larvæ can live a long time within foreign-body tubercle-like structures without increasing in size. Capsule formation is at the stage attained in other tissues within three weeks. It is unfortunate, however, that no information is available about the maximum survival period of *living* larvæ in the tissues of experimental animals.

The exact route by which the larvæ reached the thalamus cannot be determined, but there can be no reasonable doubt that it was by the arterial blood. It had never seemed likely that spontaneous human ascaris infection could reproduce the results of massive feeding of eggs or intra-carotid injection of larvæ into animals (Fülleborn, 1921*a*), although infection of the human nervous system by another nematode, *Trichinella spiralis*, has been reported (Frothingham, 1906; Hassin and Diamond, 1926; Most and Abeles, 1937).

The marked encapsulation of the parasite in our patient is at variance with the findings in the animal experiments, but resembles that found by Bunzl around the nematodes in the mole brain and in Greenfield and Pritchard's cases of cerebral schistosomiasis. The absence of neutrophil and eosinophil leucocytes indicates the chronic non-progressive state of the granuloma which is further suggested by the dense collagenous core, the absence of young reticulin fibres at the periphery and the prominence of plasma cells. The paucity of fresh glial fibres in the surrounding tissues testifies to the effective sealing off of toxic products, if such there be. Large multinucleated giant cells have been reported before both in association with larvæ (López-Neyra, 1948) and around ova (Yamauchi, 1923-24).

The perivascular hæmorrhages are present in areas remote from the inflammatory lesions and in places favoured in experimental animals, *e.g.* surface of cerebellum. They were frequently confined to the perivascular space (His') which they often filled for a considerable distance, sometimes extending into the adjacent tissues. Clotting occurred both in the extravasated blood and within the vessel so as to line the lumen. This parietal location of fibrin suggests that circulation was in progress at the time of clotting. While these appearances and possibly the presence of the red blood cells in the cerebro-spinal fluid during life, indicate ante-mortem hæmorrhage, the latter clearly occurred after the arrival of the larva in its final location and was presumably due to a later migration of parasites.

The distension of the perivascular space by globular and foamy-looking, serous material is possibly related to a derangement of the cerebral circulation consequent upon embolic occlusion by larvæ.

The non-suppurative encephalomyelitis lesions follow closely the pattern of acute anterior poliomyelitis. The distribution of the lesions, including involvement of the brain stem, substantia nigra and motor cortex, is characteristic; the restriction of severe changes to the nerve cells, the total destruction of some neurones while adjacent ones remain normal or only mildly affected, the synchronous progression of neuronophagia of the fatally affected cells, the interstitial microglial and paravascular nodules and the perivascular and meningeal exudates are all highly typical of poliomyelitis and cannot be referred to any other disease process. While it is true that in cerebral trichinosis, *microglial nodules* may be disseminated throughout the central nervous system (Hassin and Diamond), in such cases the brain and spinal cord appear to have been invaded by numerous larvæ. Taken individually many of the changes seen in the nervous system resemble those described by Rachmanow (1914) in guinea-pigs receiving injections of perienteric fluid of ascaris, but the pattern and distribution of the lesions taken as a whole make poliomyelitis a much more attractive diagnosis.

Thus it seems likely that this child suffered a clinically silent or at least undetected migration of ascaris larvæ through the cerebral vessels which continued until death. Only one or at most a few larvæ penetrated the brain and the only one seen was rendered harmless by efficient encapsulation and gave rise to no local symptoms. Superimposed upon this larval migration there was almost certainly an *infection with a poliomyelitis virus of sufficient severity to cause* death.

### (b) *Relationship of larval migration to genesis of poliomyelitis*

Shope (1941*a*) demonstrated that both the swine-influenza virus and the *Hæmophilus influenzæ suis* are necessary for the production of swine influenza, the virus alone causing much milder respiratory infection known as "filtrate disease". Swine may be infested with lungworms which carry the virus of swine influenza in a masked form (Shope, 1941*b*). Under these conditions there is no disease unless some outside stimulus is applied. The natural stimulus appears to be a seasonal change in climatic conditions (Shope, 1943*b*) but Shope regularly produced swine influenza in these animals by repeated intramuscular injections of living cultures of *Hæmophilus influenzæ suis*, while the injection of killed cultures resulted in "filtrate disease". Shope considered this stimulus to be quite non-specific and possibly an allergic or sensitisation phenomenon. The only other method which was markedly effective was the feeding of swine ascaris eggs (Shope, 1943*a*); the resulting migration of ascaris larvæ was followed by "filtrate disease" in 16 per cent. of cases. Thus there is a precedent for facilitation of a virus infection by migration of ascaris larvæ.

In recent years there has accumulated an impressive body of clinical evidence suggesting that resistance to poliomyelitis infection is lowered by certain traumatic and immunising procedures. Such evidence has been fully reviewed by Findlay and Howard (1950) who also showed that such a phenomenon can be demonstrated experimentally in mice infected intra-cerebrally with the Lansing poliomyelitis virus. In the present case the migration of ascaris larvæ may have facilitated poliomyelitis infection by inducing an immune reaction, as suggested by Shope in "filtrate disease", or by

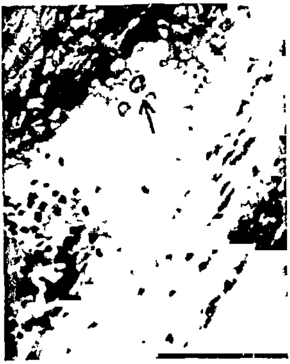

FIG. 10.—Globus pallidus showing globular fragments (arrow) of myelin sheaths free in perivascular space. Loyez' myelin stain. ×300.

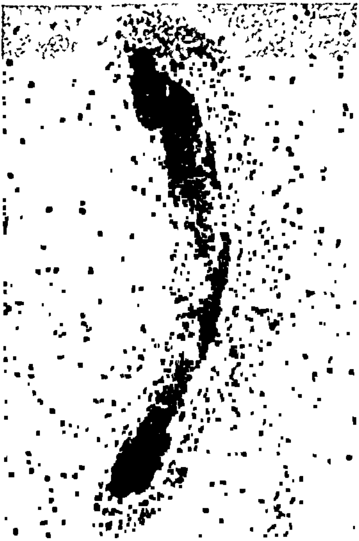

FIG. 11.—Hippocampal gyrus showing perivascular "sheath hæmorrhage" with intravascular parietal clotting. Phosphotungstic acid-hæmatoxylin. ×275.

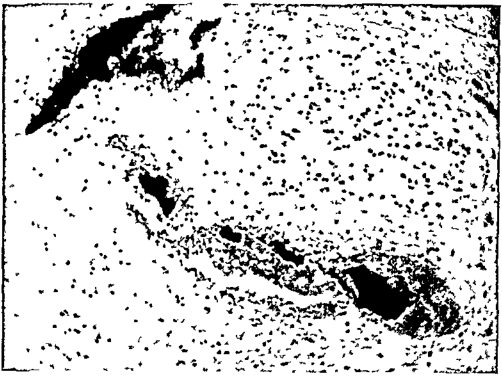

FIG. 12.—Same section as fig. 11 showing disruption of nervous tissue by hæmorrhage and clotting of extravascular blood. Phosphotungstic acid-hæmatoxylin. ×188.

CEREBRAL ASCARIASIS AND POLIOMYELITIS

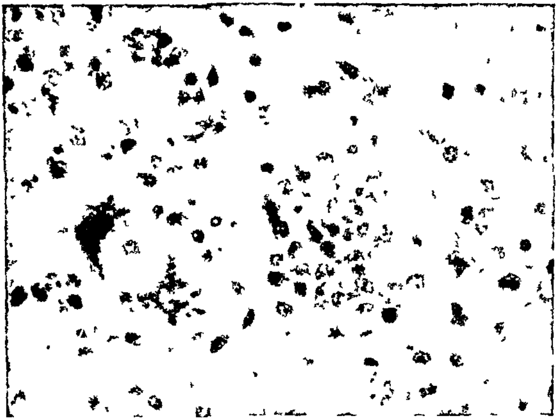

FIG 13 —Fourth cervical segment showing a normal looking anterior horn cell in proximity to a severely shrunken cell whose contents have undergone eosinophilic changes Between these two cells is a microglial nodule Leishman ×525

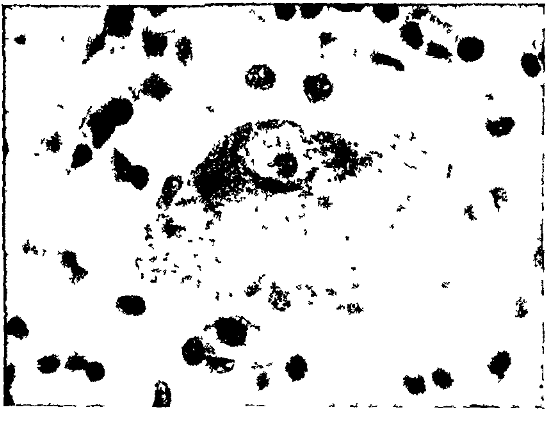

FIG 14 —Same section as fig 13 showing a chromatolytic anterior horn cell Thionin ×950

a non-specific traumatic effect, as described by Findlay and Howard, mechanical damage being inflicted upon the respiratory and alimentary systems by the migration. Additional evidence for the operation of a traumatic effect in our case is the heavy incidence of the inflammation upon the brain stem and cervical cord.

Although the virus of lymphocytic choriomeningitis may be carried into the central nervous system by *Trichinella spiralis* (Syverton *et al.*, 1947), a similar mechanism appears less likely in our case as there are no poliomyelitis lesions near the encapsulated larva or in the regions through which the larvæ appear to have passed.

The idea that ascariasis may predispose to poliomyelitis is not new, having been suggested by Martini (1937) who had noted that children with infantile paralysis seemed to harbour round worms more frequently than did other children.

### (c) *Cause of neurological symptoms in ascariasis*

As stated in the introduction, neurological symptoms in ascariasis are protean and more than one mode of production may be envisaged. While, as seems likely in our case, some neurological illnesses in patients with ascariasis are independent diseases, the dramatic recovery which sometimes follows the expulsion of the adult worms is not explained. The idea that irritation of the intestines by adult ascarids can have a *reflex* effect upon the central nervous system (Kuchenmeister and Zürn, 1881 ; Schachter, 1934) does not explain the changes in the cerebrospinal fluid. Numerous experiments reviewed by Girges (1934) demonstrating the *toxicity* of extracts from round worms, injected parenterally, have been performed, notably by Brinda (1914), but there is no convincing clinical or experimental evidence that adult worms exert a toxic effect in areas remote from the alimentary tract of the host. Attempts to demonstrate toxins in the cerebrospinal fluid of patients with " verminous meningitis " have not been successful even when the fluid showed an increased protein content and pleocytosis (Abel and Brenas, 1925 ; Dutrey and Velluz, 1939). On the other hand sensitisation, as exemplified by a reaction to intra-dermal injection of ascaris extracts, is not infrequent in patients with chronic ascariasis who may have urticaria and eczema, which sometimes disappear after evacuation of worms from the intestine.

The effects of migration of larvæ through the vessels of the central nervous system do not appear to have been seriously considered, partly because larvæ have not previously been seen in the human brain and partly because larval development only follows the ingestion of ripe ova and would hardly be affected by the expulsion of adult worms, an event so often followed by rapid recovery. It is possible, however, that larval migration is responsible for occasional cases of focal or generalised epilepsy.

## Summary

1. An encapsulated ascarid larva was found in the thalamus of a child who died with clinical and morbid anatomical evidence of poliomyelitis.

2. There was histological evidence of migration of larvæ through the cerebral vessels.

3. The poliomyelitis principally affected the cervical segments of the spinal cord and the nuclei of the brain stem.

4. The manifestations of larval migration of ascaris through the brains of animals, the possible relationship between the larval migration and the development of poliomyelitis and the cause of neurological symptoms in ascariasis are discussed.

Our thanks are due to Dr J. G. Greenfield for his help and advice in preparing the paper and to Dr N. O. Richards for her kindness in allowing us to use the clinical notes.

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616—008 . 9—095 . 1 : 616 . 36—002 . 4—092 . 9 (Rattus)

# INTERACTIONS BETWEEN ENVIRONMENT AND DIET IN THE PRODUCTION OF ACUTE LIVER NECROSIS IN THE RAT

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(Plate CXII)

Two papers on the effect of environment on liver necrosis form a background to this work. In the first Naftalin and Howie (1949) describe nutmeg liver with centrilobular necrosis proceeding to central and periportal cirrhosis in suckling and weanling pigs reared in a cold and damp environment. This lesion differs from the dietetic necrosis described by György and Goldblatt (1939) and Glynn and Himsworth (1944) and from that described by Gillman *et al.* (1945) in young rats given maize meal porridge and sour milk. Naftalin and Howie suggest that a vascular disturbance arising either within the liver itself or in the general venous system might be the cause of the pig lesion. The second paper is that of Rao (1948). Working in Bombay and using the methods of Himsworth and Glynn (1944-45), he failed to produce acute dietetic necrosis and suggested that England's colder climate, particularly in winter, might be a determining factor.

The manner of feeding the animals also warrants investigation. Weichselbaum (1935) studied the effect of Sherman and Merrill's (1925) cystine-deficient diet on 8-10-week-old rats weighing 50-65 g. In these experiments, except during the first few days when food was supplied in excess, Weichselbaum gave 8 g. to each rat per day, this being (p. 364) "near the maximum of any one rat" and "regularly consumed except by one animal". Weichselbaum considered that the food intake of his rats, which were caged separately, was restricted. He abandoned his experiments because of the high mortality associated with "hæmorrhages" in the liver after the sixth week. The ration given by Himsworth and Glynn, irrespective of the composition of their different diets, was also 8 g. per day per rat of 120-150 g. It will be seen from their tables I, II and IV (pp. 102, 104 and 108) that the average food intake per rat per day was between 4·23 g. and 8 g., depending on the composition of the diet. In only 2 diets of the 23 tested were the 8 g. eaten entirely. Himsworth and Glynn caged their rats in groups of 10, a method of management which makes it impossible to find out the exact amount of food eaten by an individual animal. Nevertheless Himsworth and Glynn stressed the importance of the exact amount of casein consumed, stating that necrosis developed only when a rat ate between 500 and 200 mg. of casein per day. They suggested (p. 103) that "smaller rats were jostled away from the feeding bowl by the bigger animals and consequently may have obtained less food" and thus might not always have been allowed to consume the protective amount of casein, *i.e.* more than 500 mg. a day. Such uncertainties about diet restriction induced me to investigate the effect on the incidence of liver necrosis of giving different amounts of the same diet and so providing a range of protein intake wider than the limits which Himsworth and Glynn considered necessary to produce the disease.

## PLAN OF EXPERIMENTS

In planning the present experiments attention was paid to the diet in the pre-experimental stage. Schwarz (1944) and György (1947) independently recognised the part played by vitamin E in diets designed to produce liver necrosis. György and Goldblatt (1949) suggested that the inclusion of unsaturated fat was an additional predisposing factor. Himsworth and Lindan (1949, p. 30), discussing the failure of some workers to produce the disease, stated that the lesion could be produced only when the diet was deficient both in

TABLE I

*Management of experimental animals, including procedure for rearing rats before experimental low-casein diets were given*

| | Experiment 1 (March-May 1949) | Experiment 2 (Nov. 1949-Feby. 1950) | Experiment 3 (Feby.-May 1950) |
|---|---|---|---|
| Mothers' diet . . . | Rowett Institute stock rat cubes +milk+green food till 16 days post-partum | Rowett Institute stock rat cubes +milk till 18 days post-partum | Rowett Institute stock rat cubes +milk till 18 days post-partum |
| Litter . . . . | 1st | 1st | 2nd from mothers used in expt. 2 |
| Preliminary high-protein diet | No. 51 (16 per cent. casein 3 per cent. yeast) | No. 82 (19 per cent. casein 3 per cent. yeast) | No. 51 |
| Age of young (days) when preliminary high-protein diet was first offered | 16 | 18 | 18 |
| Age of young (days) at weaning | 24 | 24 | 24 |
| * Weaning weight (g.) . | 32·7 | 36·2 | 42·0 |
| Low-protein diet . . | No. 47 (8 per cent. casein 3 per cent. yeast) | No. 47 | No. 47 |
| Age of young (days) when low-protein diet was first given | 36-40 | 34 | 38 |
| Duration of experiment (*i.e.* no. of days from giving low-protein diet to killing survivors) | 53 | 69-71 | 70 |

In experiment 1. Control rats were given stock diet at 33-37 days of age.
In experiment 2. Control rats were given stock diet at 24 days of age (weaning).
In experiment 3. Control rats given stock diet were not included.

* Standard deviation of weaning weights = ±12 g. based on 158 degrees of freedom.

cystine and vitamin E, and that the explanation of the latent period, *i e* the period between first giving the deficient diet and the onset of the disease, might be that the animal body " held tenaciously to its tocopherol stores " To ensure that the animals would have low reserves of vitamin E, weanling rats were reared and used in three experiments as shown in table I In order to study the effects of the amount of protein eaten, uncomplicated by individual differences in carbohydrate digestibility, a quickly absorbable carbohydrate, sucrose, was chosen instead of maize starch, as used by Himsworth and Glynn Three experiments were performed, the first in March May 1949, the second in November 1949 February 1950 and the third in February-May 1950 Males only were used because Weichselbaum reported a much higher incidence of " hæmorrhages " in this sex The rats were albinos of the Wistar Glaxo strain bred from a stock supplied by the Agricultural Research Council's Field Station, Compton, Newbury, Berkshire

TABLE II

*Details of experimental environments*

| Environment no | Experiment 1 (March May 1949) | Experiment 2 (Nov 1949 Feby 1950) | Experiment 3 (Feby May 1950) |
|---|---|---|---|
| 1 | Old animal house (brick building)<br>Usual range 63 70° F<br>Extreme range 57 80° F<br>(Groups A, B and C) * | New animal house (insulated building thermostatically controlled)<br>Usual range 70 74° F<br>Extreme range 54 74° F<br>(Groups A B, C and D) | New animal house (insulated building, thermostatically controlled)<br>Usual range 70 74° F<br>Extreme range 70 74° F<br>(Group A) |
| 2 | Rabbit hutch facing north<br>Usual range 40 50° F<br>Extreme range 34 65° F<br>Animals protected from rain but exposed to wind from which they could shelter by going to back of cage<br>(Groups D, E and F) | Unheated wooden shed<br>Usual range 40 50° F<br>Extreme range 28 58° F<br>Temperature varied according to climatic conditions Animals exposed to draughts<br>(Groups E F and G) | Heated wooden shed<br>Usual range 60 65° F<br>Extreme range 55 76° F<br>(Groups B and E) |
| 3 | | | Unheated wooden shed as in experiment 2<br>Usual range 40 50° F<br>Extreme range 26 68° F<br>(Group C) |
| 4 | | | Rabbit hutch facing north as in experiment 1<br>Usual range 35 45° F<br>Extreme range 20 60° F<br>(Group D) |

* For explanation of groups see tables VI VIII

The nature of the environments is detailed in table II and under " results " Air temperatures were recorded continuously on 7 day thermographs. Rats were weighed twice weekly throughout and at death, but growth curves are not given because they are not relevant to the questions discussed. Water was provided *ad lib.* Composition of the diets is given in table III. The food intakes of selected rats were measured daily.

TABLE III

*Percentage composition of diets used*

| Constituent | *Preliminary high-casein diet no. 51* | *Experimental low-casein diet no. 47* | *Preliminary high-casein diet no. 82* |
|---|---|---|---|
| Casein (vitamin-free: Glaxo) . | 16 | 8 | 19 |
| Yeast * . . . . . . | 3 | 3 | 3 |
| Lard . . . . . . | 7 | 7 | 7 |
| Sucrose . . . . . . | 68 | 76 | 65 |
| McCollum's salt mixture 185+KI† | 4 | 4 | 4 |
| Cod-liver oil (B.P.) (c.c.) . . | 2 | 2 | 2 |

The following B vitamins (mg. per 100 g. diet) were added to all the above diets: aneurin 0·3, riboflavin 0·3, Ca pantothenate 2·0, pyridoxin 0·3, inositol 0·5, nicotinic acid 4·0.

* Dried yeast powder (Pulv. saccharomycetis cerevisiæ exsicc.) The Pharmaco-Chemical Products Ltd., London and Burton-on-Trent.

† McCollum's salt mixture 185+0·221 g. KI per kilo salt. $I_2$ content of final diet = 0·9 mg. per 100 g.

The food intakes for all three experiments are shown in tables IV and V and particular points are illustrated in figs. 6 and 7. It will be observed from tables II and IV that the food intake of rats fed *ad lib.* is related to the environmental temperature, and to the age of the rat. The relation of food intake to the incidence of liver necrosis is shown in table VI and is considered in the discussion.

All rats were examined *post mortem.* In those which died with acute massive necrosis of the liver the organ was mottled with patches of dull grey and depressed red areas (figs. 1 and 2). Histologically, acute necrosis usually of the massive type was present (figs. 3 and 4). Hæmorrhagic lesions were also found in the lungs, stomach and thymus of some animals. In rats which died without massive necrosis, the liver was dark red in colour and small in size. Testicular degeneration was found in every rat given the experimental diet. Degenerative changes were often seen in the renal tubules but these lesions bore no relation to the environment.

## RESULTS

### *Experiment* 1

*Environments.* Two environments were used: (1) Old animal house. This room was part of a non-insulated brick building and measured 11×10×14 ft. high. It was heated by hot-water radiators and by electrical tubular heaters placed near the floor. Lighting was from ceiling windows. (2) Outside rabbit hutch facing north. This was made of wood with a roof covered with bituminised felt. It was divided into compartments 16×24×19 in., into each of which

TABLE IV—*Incidence of liver necrosis in and food consumption of rats fed* ad libitum *in experiments nos. 1, 2 and 3*

| Expt no | Group * | Rat no. | Acute liver necrosis | Average food consumption (g /rat/day) | | | Ratio of food eaten *ad lib* /restricted | | Remarks |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 1st week of experimental diet | last week of experimental diet † | till death (D) or killed (K) (no of days in parenthesis) | 1st week | till death | |
| 1 | A | 6 | + | 6·1 | ... | 6·1 (*D.* 8) | 1·15 | 1·15 | |
| | | 35 | + | 5·7 | ... | 5·7 (D. 8) | 1·09 | 1·09 | |
| | D | 8 | – | 11·8 | 14·0 | 11·9 (K. 53) | 1·43 | 1·31 | 4 rats *in* rabbit hutch, experiment 1, group E (restricted) died in 3, 6, 8 and 9 days without liver necrosis. Environmental temperature *about* 40°F. |
| | | 16 | – | 11·1 | 12·7 | 11·9 (K. 53) | 1·36 | 1·31 | |
| | | 34 | – | 12·5 | 12·5 | 11·5 (K. 53) | 1·25 | 1·25 | |
| 2 | A | 3 | + | 4·7 | ... | 4·2 (D. 36) | 1·28 | 1·13 ‡ | |
| | | 7 | – | 4·0 | 4·6 | 4·0 (K. 70) | 1·06 | 1·10 ‡ | |
| | | 35 | + | 4·4 | ... | 3·9 (D. 61) | 1·18 | 1·07 ‡ | |
| | | 37 | + | 4·6 | ... | 3·6 (D. 33) | 1·21 | 0·97 ‡ | |
| | E | 5 | + | 9·4 | ... | 9·3 (D. 25) | 1·22 § | 1·09 | Environmental temperature at death 50° F. |
| | | 10 | – | 9·8 | 11·8 | 10·9 (K. 70) | 1·27 § | 1·22 | |
| | | 31 | + | 10·0 | ... | 10·5 (*D.* 21) | 1·3 § | 1·23 | Environmental temperature at death 50° F. |
| | | 86 | – | 10 6 | 14·5 | 12·4 (K. 70) | 1·11 § | 1·32 | |
| | | | | | | | | | 3 *of* 10 *rats in* unheated wooden shed (group F, restricted) died in 5, 46, 67 days without liver necrosis. Ratios *ad lib.*/restricted for their last week of life were 1·41, 1·34 and 1·35. Temperatures 38, 28 and 40° F. |
| 3 | A | 104 | + | 4 9 | ... | 4·8 (D. 32) | no restricted group | | |
| | | 105 | + | 5·1 | ... | 4·6 (*D.* 39) | | | |
| | | 117 | + | 5·6 | ... | 5·3 (D. 40) | | | |
| | | 127 | – | 4·8 | 6·6 | 5·1 (K. 70) | | | |
| | B | 102 | – | 6·7 | 9·5 | 8·1 (K. 70) | 1·6 | 1·54 | |
| | | 107 | + | 6·3 | ... | 6·2 (D. 24) | 1·6 | 1·26 | |
| | | 119 | + | 7·9 | ... | 6·9 (D. 15) | 1·6 | 1·46 | |
| | | 125 | + | 7·9 | ... | 7·2 (D. 25) | 1·5 | 1·38 | |
| | C | 103 | – | 9·3 | 13·5 | 12·1 (K. 70) | no restricted group | | |
| | | 106 | – | 9·6 | 14·7 | 12·6 (K. 70) | | | |
| | | 116 | – | 9·0 | 15·5 | 13·0 (K. 70) | | | |
| | | 120 | – | 8·6 | ... | 8·7 (D. 11) | | | Died 11th day without liver *necrosis* |
| | D | 101 | – | 9·7 | 15·0 | 12·7 (K. 70) | no restricted group | | |
| | | 108 | – | 8·7 | 14·9 | 13·0 (K. 70) | | | |
| | | 118 | – | 11·1 | 16·1 | 13·4 (K. 70) | | | |
| | | 128 | – | 11·9 | 15·6 | 14·3 (K. 70) | | | |

* See tables II and VI for the management of the various groups. † Last week in experiment 1 = 47th-53rd day. Last week in experiments 2 and 3 = 64th-70th day.
‡ Calculated on *basis of food* offered to restricted group for period, *i e.* 3 8 or 3·6 g /rat/day.
§ Omitting rat no. 24 (see remarks column of table V) which died when not consuming all food offered.
... = *No observation* because rat already dead.

four cages could be put. The rats were thus protected from the rain, but exposed to the wind from which they could take shelter at the back of the cage.

*Methods.* Forty-three male rats, first litters born between 12th and 16th February 1949, from eleven mothers, were distributed by litter mates at random on 15th March, when 27-31 days old, into six groups. Three of these groups, labelled A, B, and C were kept in the heated animal house at 57-80° F. throughout the experiment. Six days later, *i.e.* from 21st March, they were put into individual cages containing wood wool. The cages measured 14×9×9 in. high and had a ½-in. mesh floor. The animals thus had access to their fæces.

On 15th March also, groups D, E, and F were acclimatised to outside temperatures of about 40° F. For this, each group was kept in one cage containing wood wool in an unheated brick building without windows or doors for six days, after which each animal was put into a separate cage with wood wool. Next day the wood wool was removed and the cages were transferred to the outside rabbit hutch where they remained until the end of the experiment on 16th May 1949, *i.e.* 53 days after the experimental diet was first given.

The mothers received the modification of the Rowett Institute stock rat cube containing 14 per cent. dried whole milk (Sengupta and Howie, 1948-49), liquid fresh milk and unlimited green food during pregnancy and until the 16th day *post partum* when diet 51 (containing 16 per cent. casein and 3 per cent. yeast) was substituted. The weanling rats of groups A, B, D, and E continued to eat this diet until 24th March 1949 (36-40 days of age) when the experimental low-casein diet 47 (8 per cent. casein and 3 per cent. yeast) was given. Groups A and D, which were allowed to eat to appetite, ate roughly 6·2 g. a day (*table IV*). Groups B and E were allowed at first 5 and 6 g. respectively per rat, once a day at 10 a.m. The amount given throughout the experiment was adjusted so that all of the animals in these groups always ate all of the food that was offered and yet continued to grow. The amounts given are shown in *table V*.

TABLE V

*Food offered to "restricted" groups*

| Expt. | Group | Amount of food offered (g. per rat per day) | Amount eaten | Remarks |
|---|---|---|---|---|
| 1 | B | 5, 6, 4·4 (see remarks) | All food eaten except towards the end of the experiment | Amount of food offered was reduced when air temperatures rose |
| | E | 6, 7, 7 and 8 g. for 4 days. 9 g. subsequently | All food always eaten | ... |
| 2 | B | 3·8 then 3·6 | Food often not completely eaten | Rats not truly restricted even with this small amount of food. Sometimes they ate only 2-3 g. a day |
| | F | 7-9·5 | All food always eaten except by rat no. 24 | Rat no. 24 died from acute liver necrosis in 25 days while eating in its last week an average of 7·0 g. per day, *i.e.* 74 per cent. of the food offered |
| 3 | E | 4-6 | All food always eaten | ... |

Group C, kept in the old animal house, and group F, kept in the rabbit hutch, served as controls; they were fed on Rowett Institute stock rat cubes from 21st March (33-37 days old) and were caged individually.

## DIETARY LIVER NECROSIS

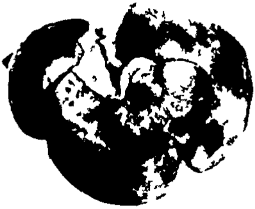

FIG 1—Acute liver necrosis Note grey (necrotic) and dark areas Natural size

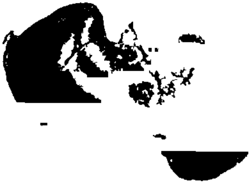

FIG 2—Normal rat liver Natural size

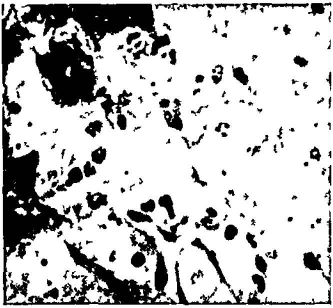

FIG 3—Acute liver necrosis Note absence of granulation of cytoplasm and presence of pyknotic nuclei Maximow's stain ×450

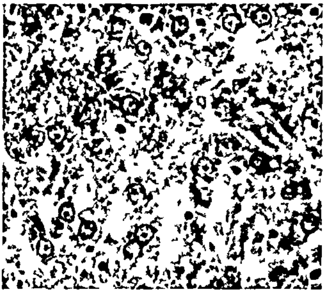

FIG 4—Normal rat liver Maximow's stain ×450

*Results.* The incidence of deaths in experimental and control rats is shown in tables VI and VII respectively and the percentage survival rate of rats in group A (diet 47 *ad lib.*) is shown in fig. 5. The results are discussed with those of experiment 2.

TABLE VI

*Incidence of deaths in experiments nos. 1, 2 and 3 in rats given experimental diet*

| Expt no | Group* | Management | | | No of rats tested | No dead with acute massive liver necrosis | Median survival time (days) | No dead without acute necrosis of liver |
|---|---|---|---|---|---|---|---|---|
| | | Housing | Experimental diet † | Feeding | | | | |
| 1 | A | Old animal house | 47 | *Ad lib.* | 8 | 7 | 17 | 0 |
| | B | ,, | 47 | Restricted | 9 | 1 ‡ | ... | 0 |
| | D | Rabbit hutch | 47 | *Ad lib.* | 8 | 0 | ... | 0 |
| | E | ,, | 47 | Restricted | 9 | 0 | ... | 4 |
| 2 | A | New animal house | 47 | *Ad lib.* | 9 | 8 | 43 | 0 |
| | B | ,, | 47 | Restricted | 9 | 8 | 40 | 0 |
| | C | ,, | 62 | *Ad lib.* | 5 | 1 | ... | 0 |
| | E | Unheated wooden shed | 47 | *Ad lib.* | 10 | 3 | ... | 0 |
| | F | ,, | 47 | Restricted | 10 | 1 | ... | 3 |
| 3 | A | New animal house | 47 | *Ad lib.* | 12 | 11 | 19 | 0 |
| | B | Heated wooden shed | 47 | *Ad lib.* | 12 | 8 | 26 | 1 § |
| | C | Unheated wooden shed | 47 | *Ad lib.* | 12 | 0 | ... | 1 |
| | D | Rabbit hutch | 47 | *Ad lib* | 12 | 0 | ... | 1 § |
| | E | Heated wooden shed | 47 | Restricted | 8 | 0 | ... | 0 |

* For groups C and F of experiment 1 and D and G of experiment 2, see table VII

† Composition of diets in table III

‡ This animal died 53 days after being first given the experimental diet For 4 days before this rat died the temperature of the animal house was around 80° F

§ The two rats indicated were litter mates and were killed on the 16th and 21st days respectively of the experiment because of severe otitis media

TABLE VII

*Incidence of deaths in experiments nos. 1 and 2 in rats fed on stock rat cube diet* ad lib.

| Expt no | Group | Housing | No tested | No died | Remarks |
|---|---|---|---|---|---|
| 1 | C | Old animal house . . | 5 | 0 | ... |
| | F | Rabbit hutch | 4 | 3 | No liver necrosis |
| 2 | D | New animal house . | 5 | 0 | ... |
| | G | Unheated wooden shed | 10 | 7 | No liver necrosis |

## *Experiment* 2

Experiment 2 was begun in November 1949 when the new animal house at the Rowett Institute was nearly completed. Certain modifications from experiment 1 were introduced affecting (1) the environment, (2) the preliminary procedure, and (3) the method of randomisation and acclimatisation to the unheated environment.

1. *Environments.* The new animal house is an insulated building designed to maintain a temperature of 70-74° F. throughout the year. This was the heated environment and except on four occasions when the temporary electrical current failed the temperature remained between 70 and 74° F. The cold environment was an unheated wooden shed 10×15×14 ft. high, lit by two side windows 2 feet square and a window 3×2½ ft. over the door. The rats were placed in individual cages in two tiers in a rack down the middle of the room. They were thus protected from wind and rain but were exposed to draughts.

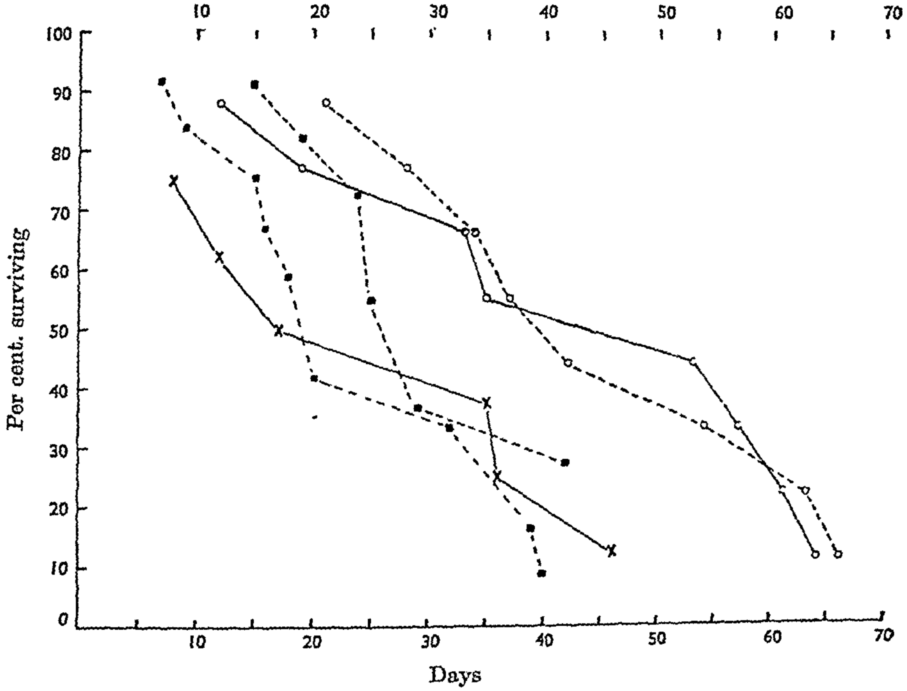

FIG. 5.—Experiments 1, 2 and 3. Percentage of rats surviving on days after receiving experimental diet:—

X ———— X : Experiment 1, group A, diet no. 47 *ad lib.* (8 rats).
o ———— o : „ 2, „ A, „ 47 „ (9 rats)
o – – – – – o : „ 2, „ B, „ 47 " *restricted* " (9 rats)
▪ —.—.— ▪ : „ 3, „ A, „ 47 *ad lib.* (12 rats)
▪ – – – – – ▪ : „ 3, „ B, „ 47 „ (11 rats)

2. *The preliminary procedure.* The mean weaning weight of 43 male rats in experiment 1 was 32·7 g. compared with 43·8 g. for male albinos of the stock colony (table I). These means differ significantly (P = 0·01). On this account in experiment 2 the protein in the preliminary diet was increased to 19 per cent. casein plus 3 per cent. yeast (diet 82) and this diet was not substituted for the stock diet till the 18th day *post partum*. In experiment 2 the mean weaning weight of 58 rats was 36·2 g., significantly greater than in experiment 1 (P = 0·05). But it should be noted that in experiment 3 where diet 51 was again used in the preliminary period, the mean weaning weight of 56 rats was 42 g., significantly greater than in experiment 2 and similar to that of the stock rats. Without direct comparisons, it is difficult to state the cause of the differences in weaning weight.

To offset the effects of better preliminary diet in experiment 2, the experimental diet was given when the rats were 34 days old, *i.e.* 2-6 days earlier than in experiments 1 and 3.

3. *Randomisation and acclimatisation.* Although the mothers were mated early in October 1949 the litters were born over a period of 4 weeks from 27th October to 22nd November. It was thus necessary at the outset of the experiment to adopt a method of randomisation and acclimatisation different from that used in experiment 1. Fifty-eight male rats from 21 litters were

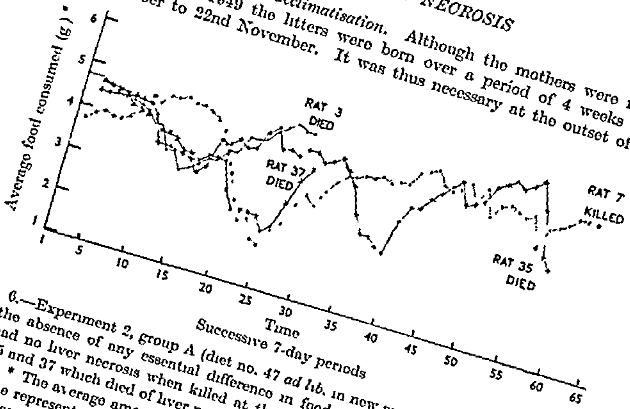

FIG. 6.—Experiment 2, group A (diet no. 47 *ad lib.* in new animal house). This shows the absence of any essential difference in food intake between rat no. 7 which had no liver necrosis when killed at the end of the experiment and rats nos. 3, 35 and 37 which died of liver necrosis.

* The average amount of food consumed is plotted against time, each point of time representing 7 days. Thus the first point on the time abscissa = days 1-7; the second, days 2-8, etc. Note that owing to differences in age each rat does not begin at the first point in time. 1 g. of diet no. 47 contained 80 mg. of casein.

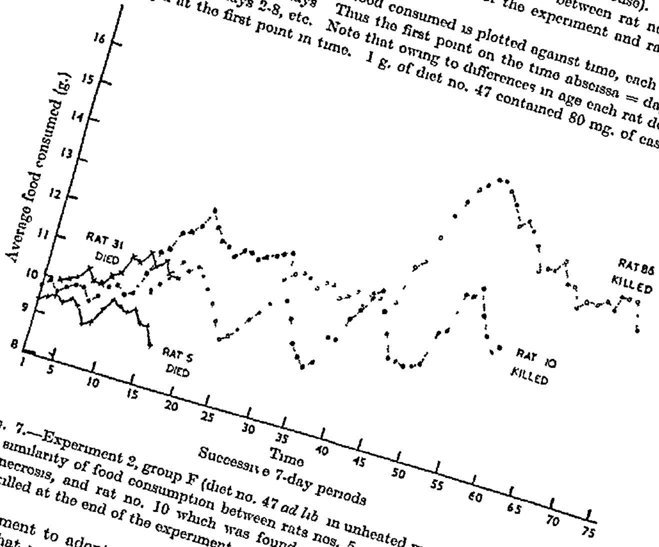

FIG. 7.—Experiment 2, group F (diet no. 47 *ad lib.* in unheated wooden shed). To show similarity of food consumption between rats nos. 5 and 31, which died with liver necrosis, and rat no. 10 which was found to have escaped liver necrosis when killed at the end of the experiment. For rat no. 86 see p. 662.

used. On weaning, each rat was allocated to one of 7 groups (table VIII) according to a scheme of random numbers drawn up from Fisher and Yates's table xxxiii (Fisher and Yates, 1948). Acclimatisation to the cold was carried out as follows: On the 28th day of life the rats allocated to the unheated wooden shed (groups E, F and G) were placed there during the day in group cages containing wood wool. At night (5 p.m. to 9 a.m.) they were returned to the heated animal house. On its 31st day of life each rat was transferred to its own cage and remained day and night in the unheated wooden shed. Wood wool was provided till the 36th day. On their 28th day of life the rats allocated to the heated animal house (groups A, B, C and D) were placed in individual cages with wood wool and remained thus caged till death or to the end of the experiment. Survivors were killed 69-71 days after the experimental diet was first given.

TABLE VIII

*Groups in experiment no. 2*

| Group | Environment | Preliminary diet *ad lib* from weaning to 34th day of age | Experimental diet when 34 days old | Feeding of experimental diet |
|---|---|---|---|---|
| A | Animal house . . . | 82 | 47 | *Ad lib.* |
| B | ,, . . . | 82 | 47 | Restricted |
| C | ,, . . . | 82 | 82 | *Ad lib.* |
| D | ,, . . . | Stock | Stock | ,, |
| E | Unheated wooden shed . . | 82 | 47 | ,, |
| F | ,, . . | 82 | 47 | Restricted |
| G | ,, . . | Stock | Stock | *Ad lib.* |

Rats distributed on weaning to groups by random numbers (Fisher and Yates's table xxxiii)

The amounts of food allowed to the restricted groups are shown in table V. Often the rats in the heated animal house did not eat even this small amount (3·8 or 3·6 g. a day) and were, therefore, not truly restricted.

*Results.* The incidence of deaths is shown in table VI and the percentage survival rate of the rats in group A (diet 47 *ad lib.*) and group B (diet 47 "restricted") in fig. 5. It will be noted that the results differ in three ways from those of experiment 1.

(1) In the heated animal house at 70-74° F. the incidence of deaths and the median survival time were not different in the "*ad lib.*" and "restricted" groups. It was not realised until the experimental diet had been given for 4 weeks that 3·6-3·8 g. of food per rat per day would not be an adequate restriction at this temperature. It was decided, however, not to interfere with the original plan. It may be worth noting that the one death in the comparable group in experiment 1 (group B, table VI) took place when the environmental temperature had risen to nearly 80° F. for 4 days.

(2) The median survival time of the animals in the heated environment in experiment 2 was 43 days as compared with 17 days in experiment 1. In the absence of a direct comparison of the two procedures using litter mates under identical environmental conditions,

it is tentatively suggested that the suckling rats in experiment 2 had a greater store of vitamin E or that the higher protein of the preliminary diet 82 increased the resistance to necrosis. It has been shown that some stock diets are near the deficiency level for vitamin E (Hines and Mattill, 1943; Bruce, 1950). However, diet 51 containing 16 per cent. casein and 3 per cent. yeast is not fully adequate for the lactating mother or the growing rat, being deficient in sulphur amino-acids, especially cystine (Sure, 1941; Mulford and Griffith, 1942), and in labile methyl groups (Salmon, 1947). Rats reared as in experiment 1, therefore, would begin their experimental period in a suboptimal nutritional state.

(3) Four of 20 rats died from acute liver necrosis in the unheated environment in experiment 2, groups E and F, as compared with no deaths from necrosis in the corresponding groups D and E of experiment 1 (table VI). The deaths from liver necrosis can hardly be ascribed to differences in food consumption between the rats which developed necrosis and those which did not develop necrosis in the same environment. At this stage it can only be pointed out that experiment 2 was conducted at a different season and that the climatic conditions were not identical.

In view of differences in results between experiments 1 and 2 a third experiment was conducted in the spring of 1950, using four gradations of temperature which by then were available.

*Stock rats* (table VII). The rats given stock diet and used as controls in experiments 1 and 2 grew well in the heated environments but fared badly in the unheated environments. Of the 14 rats in the unheated environments 10 died. The cause of death was undetermined, but in no rat was liver necrosis or pneumonia found. Food consumption was greater in the unheated environment, but growth was considerably less. This is in marked contrast to the rats fed on the deficient diet 47, where food consumption was greater and growth considerably better in the cold than in the heated environments.

## *Experiment 3*

*Environments.* Environment 1 was the same as environment 1 of experiment 2—the new animal house with a temperature of 70-74° F.; environment 2 was a wooden shed 10 × 15 × 14 ft. high, lined with asbestos boarding and heated by three electrical tubular heaters thermostatically controlled. Throughout the major part of the experiment a temperature of 60-65° F. was maintained. This simulated the conditions of the old animal house of experiment 1 but the variations in temperature were much less; environment 3 was the unheated wooden shed of experiment 2, and environment 4 the rabbit hutch used in experiment 1.

The diets used were nos. 51 and 47 as in experiment 1. No attempt was made in this experiment to compare the effects of different preliminary procedures.

*Methods.* Fifty-six male rats born between 1st and 21st January 1950, second litters from 15 mothers, were used. They were divided into 4 "*ad lib.*" groups of 12 rats each (A, B, C and D), and one "restricted" group of 8 rats (E).

This latter group was placed in the environment of 60-65° F. which simulated the conditions at the beginning of experiment 1.

The procedure, shown in table I, followed that used in experiment 1, with randomisation by litter mates. Acclimatisation to the cold environments was carried out as in experiment 2. Experimental diet 47 was commenced when the rats were 38 days old. Survivors were killed 70 days after the experimental diet was first given.

*Results.* The results confirmed those of experiment 1. The incidence of deaths is shown in table VI and the percentage survival rates in fig. 5. The food consumption of selected animals is summarised in tables IV and V.

## DISCUSSION

From these results two conclusions may be drawn: (1) environment plays a definite part in determining the incidence of acute liver necrosis in rats given a low-protein, vitamin E-deficient diet, and (2) the method of feeding is important.

### *Environment*

The most obvious difference between the environments was that of the air temperature. The effects of fresh air as compared with a static atmosphere, and of differences in humidity, barometric pressure and other variables were not determined in these experiments.

In the old animal house in experiment 1, the temperature fluctuated between 57° and 80° F., being nearer 63° F. at the beginning of the experiment. In experiment 2 it was intended to hold the temperature of the new animal house steady at 70-74° F., but owing to failure in the temporary electrical supply this was not possible until the latter part of the course when the building was completed. The first three deaths of experiment 2 occurred in animals of different ages on the same night of 19th-20th December, when the temperature dropped suddenly from 74° F.-58° F. It became clear as this experiment progressed, and from experiment 3, that deaths occur regularly if a steady and sufficiently high environmental temperature is maintained; but it is not yet certain whether a fluctuating temperature or a sudden drop in temperature may precipitate necrosis. Dent (1947, p. 316) also noted that some of his rats "happened to die on the same day with acute necrosis of the liver" after they had been 118 days on the experimental diet but he does not comment on his environmental conditions. The best environmental temperature for producing the disease in my experiments was 70-74° F. though until further information is available about the effect of still higher temperatures this should not be regarded as the optimum.

The results of exposing rats to cold differ from those described by Naftalin and Howie for the pig. The pig's liver contains a large amount of smooth muscle in its hepatic venous tree (Naftalin and Howie), the muscle being present not only in the large radicles of the hepatic vein but sometimes at the junction of the central and sublobular veins. The rat's liver is devoid of such musculature, which possibly plays a part in producing the centrilobular necrosis in pigs exposed to cold and damp. Moreover the environmental conditions in Naftalin and Howie's experiment with pigs were not the same as in the present experiments. The pigs lay with their abdominal walls on cold concrete. The rats were subjected to a cold climate but their abdominal walls were not more exposed to chill than the rest of their bodies.

The reasons for Rao's inability to produce the disease in Bombay are not apparent but further work using higher environmental temperatures with high relative humidity such as occurs in Bombay might give the explanation.

### *Manner of feeding*

The other purpose of these experiments was to test the validity of Himsworth and Glynn's statement (p. 103) that it is the amount and not the proportion of protein consumed that is of importance, that consumption of from 200-500 mg. of casein per rat per day leads to necrosis, that the consumption of more than 500 mg. of casein per rat per day protects and that the further addition of yeast protein enhances the protective properties of the diet (p 106).

From their published figures a diet containing 8 per cent. of casein (*i e.* diet 47 of the present work) should lead to acute massive necrosis in about 10 per cent. of the rats This amount of casein (8 per cent.) was chosen in my experiments because of Weichselbaum's statement (p. 364) that about 8 g. of food per day (equivalent in my experiments to 640 mg. of casein) was eaten by animals of 50-65 g. and was "near the maximum of any one rat". An allowance of 4-6 g. per rat per day (table V, groups 1B and 3E) should therefore restrict food intake in the sense of the animals being offered less than they would eat if fed *ad lib.* This expectation was supported by the observation that in these groups the animals quickly ate all the food offered. These "restricted" rats consumed 320 480 mg. casein plus 120-180 mg. yeast (approx. 50-75 mg. of yeast protein) per day, *i e* they ate within the limits which according to Himsworth and Glynn should have led to necrosis. On the other hand the rats fed *ad lib.* (groups 1A, 2A, 3A and 3B) if Himsworth and Glynn's hypothesis was correct, should have eaten enough to be protected against necrosis. The results of experiments 1 and 3 (table VI) of this paper do not however support their hypothesis In these experiments, the rats which ate *ad lib.* in the heated environments with an air temperature of about 65° F. died from acute massive necrosis—the "restricted" did not On the other hand, in experiment 2 when the temperature was 70-74° F. the rats in the "restricted" group B did not always consume the small amount of food they were offered (3 6 or 3 8 g. per rat per day) and were thus eating according to appetite for part of the time. The survival rates were then not different from the "*ad lib.*" group A (table VI). It will be noted that the ratios of the food intake of the "*ad lib.*" to the "restricted" groups in the first week of the experiment were 1 15 and 1 09 in experiment 1; and 1·28, 1 06, 1 16 and 1 12 in experiment 2 (table IV). Thus at the beginning of the experiments the ratios differed little, but the results were different. Whether in an environmental temperature of 70-74° F. a greater restriction of food would prevent necrosis has yet to be determined. If this should be so it is likely that the degree of restriction required to prevent necrosis at this higher temperature will be greater than at a lower temperature, because low temperature is in itself protective. Moreover in the unheated environments seven rats died without liver necrosis (table VI, groups 1E and 2F). After the first four days of experiments 1 and 2 food restriction below 8-9 g. per rat per day in the unheated environments was not attempted because it was realised that less food than this (*i.e.* a ratio of food intake "*ad lib.*"/"restricted" greater than 1·3 to 1) at an environmental temperature of 40° F. or lower was insufficient to prevent the rats from dying suddenly without liver necrosis. It should be noted that rat 24 of group 2F (tables V and VI) died with liver necrosis at an environmental temperature of 52° F. when not eating all the food which it was offered, *i.e.* it was not "restricted". These studies indicate that a rat survives or dies of liver necrosis or of inanition according to interactions between the environment, the diet and the manner of feeding.

While it may be argued that low environmental temperature may protect by increasing the food intake, reference to table IV and fig. 7 (experiment 2) shows that rats 5 and 31, which died from acute necrosis, differed very little in their food consumption from rat 10 which survived the experiment. The figures for rat 86 which was younger and therefore exposed to the cold environment at a later date, are not directly comparable. Nevertheless for a similar period, *i.e.* the first 4 weeks of its life, its food consumption was not different. These four rats had approximately the same weight. It does not seem therefore that liver necrosis is prevented by a low environmental temperature solely because the low environmental temperature increases food intake. Again, while low protein intake may be a conditioning factor, the figures that are available, while not sufficient to provide results that can be analysed statistically, do not support the view that low voluntary food consumption *per se* is a cause of necrosis. Thus, in experiment 2, rats 3, 35 and 37 had average food consumptions of 4·2 g., 3·9 g. and 3·6 g. per day and suffered necrosis, while rat 7 with an average food consumption of 4·0 g. per day did not (table IV and fig. 6). All four animals had a fluctuating intake which was not related to the environmental temperature. Fig. 6 shows that 2 rats died while increasing their food intake. In experiment 3, group A, rats 104, 105 and 107 had also low voluntary food consumptions of 4·8 g., 4·6 g., and 5·3 g. and suffered necrosis, while rat 127 with a food consumption of 5·1 g. did not suffer necrosis (table IV). In group B of this experiment a similar relationship exists between rats 107, 119 and 125, and rat 102. In experiment 3 a similar fluctuating food consumption was recorded. Thus observation of food consumption is not sufficient to predict which rats will die of necrosis.

Furthermore, in experiment 2, group C (diet 82, 19 per cent. casein, 3 per cent. yeast), one rat among 5 died from acute liver necrosis on its 49th day of age (table VI). The food intake of this particular rat was not measured, but as its growth curve paralleled those of the other four rats in the group, one may assume that its food consumption was not different. All this evidence would seem to point to a fundamental change in metabolism rather than in protein consumption or a simple protein or vitamin-E deficiency as the cause of liver necrosis.

Two differences between these experiments and those of Himsworth and Glynn should be pointed out. First, the rats used in my experiments were males weighing about 70 g. at the beginning of the experiment in their 5th or 6th week of life; those of Himsworth and Glynn were of either sex and of 120-150 g. in weight. As the food intake of a rat is a function of its body weight, the largest amount, namely 6 g., offered to a "restricted" group in a heated environment would have meant even greater restriction if I had used heavier rats of the weights employed by Himsworth and Glynn. It is unlikely, therefore, if larger animals had been used in my experiments that the results between "restricted" and "*ad lib.*" fed groups would have been different. Secondly, Himsworth and Glynn used corn (maize) starch as the carbohydrate, while my diets 51 and 47 contained sucrose. Sucrose and corn starch are not nutritionally equivalent but in the absence of a direct comparison it is not possible to say whether different carbohydrates would influence the outcome.

## *Hypotheses*

From my experiments it is obvious that a simple explanation for the dietary cause of this disease is not yet available The results show that cold and restriction are somehow capable of protecting rats against acute dietary necrosis of the liver Unfortunately the mode of action cannot as yet be explained but some speculations may be offered, especially in the light of hypotheses discussed by *György, Rose, Tomarelli and Goldblatt (1950) in their most recent* analysis of this disease

These workers found that rats given diets containing British yeast (D C L vitamin $B_1$ yeast of the Distillers' Company Ltd, Glasgow) were more likely to develop necrosis than rats given diets containing American yeast (Type 50 brewers' yeast, Standard Brands Inc ) They concluded that a pure deficiency of cystine and vitamin E does not cause the disease, and suggested two possibilities (1) the presence of some unidentified toxic factor in British yeast, and (2) the production of toxic metabolites In my experiments an unidentified toxin in the yeast is not an acceptable explanation because the rats which ate most of the diet, i e those kept in the cold, escaped the disease, a result not to be expected if the diet were itself toxic On the other hand, cold and dietary restriction might act similarly by leading to more complete metabolism which might be less likely to favour the production and accumulation of metabolites toxic to the liver Yet a third possibility may be suggested, metabolic imbalance, which does not raise the question of toxin or toxic metabolites The liver cell appears to be kept intact by balanced forces controlling its metabolism in one direction or the opposite It is not suggested that these forces act at one point only in the metabolic chain Should any of the forces acting in the same direction, singly or in conjunction, be weakened, the metabolic processes become unbalanced However, before necrosis can occur another force acting in the opposite direction may be necessary

Two substances acting in one direction have definitely been identified, namely, cystine and vitamin E The work of Schwarz and of Hove and Harris (1947) suggests that xanthine may be added to the list though this substance has not yet secured such general acceptance as an inhibitor of dietary necrosis as have cystine and vitamin E Weichselbaum prevented the deaths of two early cases of cystine deficiency in rats by injecting intraperitoneally 150 mg of glutathione Leaf and Neuberger (1947) found that this substance rapidly disappears from liver tissue in rats fed on a low protein diet (100 mg of yeast = 40 50 mg of yeast protein per rat per day)

Should the effect of these protective substances become too great, necrosis will again result, though the metabolic processes leading to this visible cellular change may be different Excess of cystine has been shown by *Curtis and Newburgh (1927) and by Earle and Victor* (1942) to lead rapidly to liver necrosis in rats Deficiency of vitamin E or excess of vitamin E both result in a marked elevation of phosphorus turnover (Weissberger and Harris, 1943) No reports have been found on the effects of excess vitamin E or xanthine on liver structure

It would also appear from a study of the literature that thyroxin

(Handler and Follis, 1948) and in certain circumstances choline (Himsworth and Glynn, 1944-45) may be substances which act in the other direction. Methionine in small amounts would also come into this group. Using oxidised casein, which is free from cystine and methionine, as a source of protein, Hove *et al.* (1949) found that necrosis did not occur in vitamin-E deficient rats unless 0·25 per cent. of methionine was added. Amounts of methionine of the order of 1 per cent. or more can also prevent liver necrosis, presumably because some of the methionine is converted to cystine. Excess methionine (6·4-12·4 per cent.), however, results in atrophy, without necrosis, of liver cells (Earle, Smull and Victor, 1942).

The "restricted" groups housed at 60-65° F. were deficient in cystine and vitamin E and it is suggested that the forces opposing these deficiencies were also reduced. "Restricted" animals cannot satisfy completely their energy requirements. It may be that the available energy-giving substances are metabolised elsewhere in the body than in the liver. In experiment 2 the environmental temperature was 70-74° F., which is nearer thermoneutrality for the rat, *i.e.* the range of about 81-91° F. within which "the environmental temperature is perfectly adjusted to keep the body temperature normal without regulation", and "the heat loss from the body is equal to the 'minimum' heat production" (Brody, 1945, p. 284). As the environmental temperature nears thermoneutrality, changes in temperature exert a diminishing effect on basal metabolic rate. For this reason, at 70-74° F., compared with 60-65° F., energy requirements are more easily satisfied, hence it was more difficult to impose a true restriction of food intake. Any excess of food beyond the energy requirements would not be diverted from the liver elsewhere nor would it provide in a deficient diet the necessary building blocks for good growth.

The rats fed on the experimental diets in the unheated environments were also deficient in vitamin E as shown by their testicular degeneration and lack of pigment in their teeth; to judge by the poor quality of their hair compared with that of rats fed on stock diet they were also deficient in cystine. Here too, the relative excess of energy-producing substances would be diverted to tissues other than the liver. Increased metabolic rate in the cold is due to increased tissue metabolism. In experiment 2 the animals which died from acute necrosis in the unheated wooden shed presumably would not have this tissue stimulation.

It will be noted that the opposing substances fall into two categories, either anti-oxidants or oxidants. The immediate problem of producing acute liver necrosis with regularity may now be resolved into a search for more of these substances, particularly those which act in opposition to vitamin E and cystine. The essential lesion would appear to be in the functioning of the liver cell and it is probably at this level that the final explanation of the necrosis should be sought. These speculations emphasise that the experimental production of this disease is still not fully understood. In the meantime the relationships between environment, manner of feeding and incidence of liver necrosis which are described in this paper indicate new methods of attack.

## SUMMARY

1. The incidence of acute liver necrosis in young male albino rats has been studied under different environmental and dietary conditions. Their diet was low in protein, high in sucrose and deficient in vitamin E.

2. The highest incidence of acute liver necrosis occurred when the environmental temperature was 70-74° F. At 60-65° F. the rats given unlimited food showed a lower incidence of necrosis and survived longer, while in the two environments with a temperature range of 35-50° F. (extreme 20-68° F.) there were no deaths from acute liver necrosis in two experiments done during the spring of the year. Three of 10 rats died in a winter experiment when housed in an unheated wooden shed with a temperature about 50° F. Temperatures higher than 74° F. have not been studied.

3. Liver necrosis was prevented in an environment of 60-65° F. by food restriction in which the ratio of food eaten by "*ad lib.*" to "restricted" groups ranged from 1·09-1·6. In restricted groups the casein consumed was 320-480 mg. per rat per day. Necrosis was not prevented at an environmental temperature of 70-74° F. with the degree of food restriction used, viz. a ratio of 1·2 at the beginning of the experiment. In the unheated environments food restriction resulted in the deaths without liver necrosis of 7 of 19 rats.

4. Himsworth and Glynn's hypothesis that the consumption of 200-500 mg. of casein per rat per day is the necessary condition for the production of acute liver necrosis is not confirmed.

I wish to thank Mr A. W. Boyne of this Institute for much helpful discussion and for the statistical design of the experiments.

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# THE ACTION OF POLYMORPHS ON HEAT-KILLED STAPHYLOCOCCI

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(Plate CXIII)

During cinematographic studies of human polymorphs by phase-contrast microscopy it was noted that a polymorph moved towards an aggregate of heat-killed and fixed staphylococci numbering many thousands (fig. 2) and detached a large raft (figs. 3-10). Hitherto, this phenomenon has not apparently attracted attention.

## Methods

*The apparatus.* A description of the time-lapse cinematographic outfit will be given in a subsequent publication by its designer and maker, Mr P. Cliffe. A few essential details and in particular a method of making a watching eye-piece are given here, since no satisfactory equipment appears to be readily available. A watching eye-piece is of great value in recording changes in living cells, even in still photography.

In cinemicrography control of vibration is the greatest difficulty; it is usually attained by massive concrete mounts. It is possible, however, to eliminate vibration by attention to two details. The camera and microscope are mounted on a common base-board, the camera in the vertical position. Extraneous vibration is eliminated by mounting the base-board on thick rubber bungs such as are used by engineers for mounting machinery. It is necessary to experiment with different thicknesses of rubber, as periodicity of vibration varies from place to place. However, extraneous vibration was eliminated on the third floor of a ferro-concrete building, at a corner site in a town.

Vibration from the camera mechanism causes serious blurring of focus. This is far more difficult to eliminate. It disappears entirely when the microscope is itself mounted on selected rubber bungs, separating it from the base-board. The watching eye-piece, as described below, is attached to the camera, its lower portion forming a sleeve around the microscope ocular; this sleeve must not touch the ocular. The microscope ocular should be of the projection type, with the top component fully extended.

Films were taken with a Cooke, Troughton & Sims phase-contrast microscope, ×95 objective, ×6 compensating ocular. Depth of focus is very small, and exact register of focus as observed and as projected on to the film is essential and highly critical.

Fig. 1 is a diagram showing the construction of a watching eye-piece. A T-tube, with a sleeve B, fits over the microscope ocular A. A hæmocytometer cover slip is "bloomed" on one side, and mounted at C at an angle of 45°,

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In order to take cinematographic records by phase contrast at high magnification certain conditions must be satisfied. All events photographed must take place on the lower surface of the cover-slip. There must be a minimum of material in the field of view; otherwise the halo produced by phase contrast obscures definition. The distance between cover-slip and slide must be the least possible. The preparation must be sealed to prevent drying and convection currents.

### *Material examined*

After many experiments the following procedure was adopted. *Staphylococcus aureus*, Oxford strain, was grown for 18 hr. on Hartley's agar; if grown for longer, leucocidin was formed and phagocytosis did not occur. The culture was liberally applied in a streak to one end of a siliconed cover-slip (see below); it was then fixed by heat.

Heparin was placed on a finger and the finger was pricked through this. A drop of blood was then placed on the cover-slip as close as possible to the streak of cocci without touching it. The cover-slip was placed in a moist chamber, and incubated at 37° C. for one hour. Many polymorphs now adhered to the cover-slip, but there were far too many red cells for cinematography. As much blood as possible was removed with a pipette, and heparinised plasma from the same subject was used to wash away as many cells as possible. The cover-slip was then pressed very firmly on to a slide; the plasma now covered the cocci. The edges were sealed with wax.

Siliconed cover-slips were prepared as follows. After heating to 400° C. they were dipped in a 3 per cent. solution of "D.C. 200" fluid (Albright and Wilson, Ltd.) in carbon tetrachloride. Excess solution was strained away, and solvent removed by heating for 30 minutes at 110° C. The cover-slip was then baked for 3 hr. at 250° C. Siliconed glass cannot be "wetted" and droplets of blood or plasma remained spherical.

Time-lapse photographs were taken by an electric timing device at intervals of from 2-8 seconds.

## RESULTS

Polymorphs in plasma remained spherical, and did not put out pseudopodia or move until they touched a surface. This surface might be glass, or an air-bubble; they attached themselves firmly to a siliconed surface, and moved freely on it. Phagocytosis was never observed in polymorphs supended in plasma; moreover when polymorphs were attached to a surface they very rarely ingested organisms free in the plasma. Cocci were repeatedly seen to float across actively moving polymorphs without being phagocytosed; yet the distance between cover-slip and slide was so small that red cells were flattened. Polymorphs did not move towards cocci which were more than 100 $\mu$ distant; but they forced their way through intervening red cells to reach the cocci. When moving, the leading edge of the polymorph was non-granular; the succeeding portion contained granules in active motion; the distal granules were motionless. Cocci were engulfed by the leading edge and instantaneously detached from the cover-slip; they moved rapidly to the centre of the polymorph and if more cocci were taken up they were all collected in one central pattern.

After 1½ hr. in sealed preparations polymorphs developed large vacuoles; they ceased to move away from their position, but sent out pseudopodia in all directions, indenting red cells. At this stage phagocytosis has been observed to continue, but only in a rare case where a coccus drifted into contact. In 2 hr. movement ceases, and disintegration of the polymorph begins.

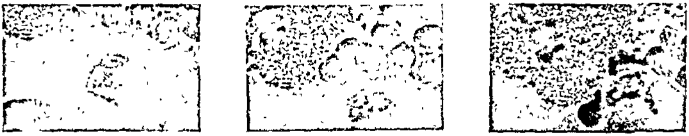
In the series of photographs illustrated, a polymorph moved towards an aggregate of cocci numbering many thousands (fig. 2). It appeared to merge with the coccal aggregate (figs. 3-6) and through the watching eye-piece could not be separately distinguished; but since only 10 per cent. of the light is deflected, definition is poor. It was then observed to extend over the whole margin (fig. 7); its three lobes were widely separated without any visible nuclear bands. Isolated cocci were phagocytosed and moved freely in a thin wall of cytoplasm. After 20 min. a cleft appeared in the aggregate (figs. 7-8) and very quickly spread until a bean-shaped raft in area about 400 sq. $\mu$ was detached. This raft became detached from the surface, and moved freely away from the aggregate (fig. 10); in this figure another polymorph is seen applied to the edge of an aggregate. Later the three lobes drew closer together; but by this time two hours had elapsed and no further observations were valid.

## *Discussion*

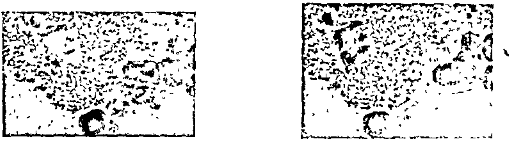
Neither in the film as projected nor by direct observation was it possible to see a cell-wall around the raft. It seems that the mass was detached by a shearing action; there must however be some unknown mechanism whereby a single coccus firmly fixed by heat to a slide is removed by a polymorph instantaneously. Such cocci adhere firmly to slides even when washed or immersed in 25 per cent. sulphuric acid. The removal of single cocci strongly suggests a solvent action exerted remarkably quickly on the surface of the coccus.

It is surprising to find that a single polymorph can remove from a surface aggregate many thousands of cocci, and carry these loose into the plasma. If this occurs *in vivo* the mass would presumably be carried in the blood stream until arrested as an embolus; and if live cocci were carried in this way metastatic suppuration might follow. However, all attempts to design a method wherein living organisms were used were unsuccessful; for example, live cocci suspended in plasma which was later clotted were not phagocytosed.

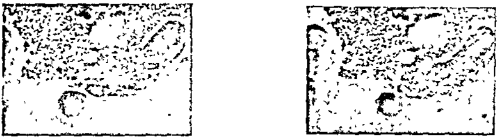
## PHAGOCYTOSIS OF STAPHYLOCOCCI



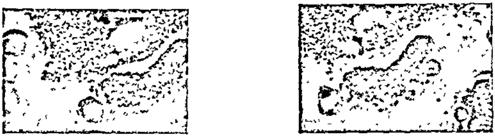
FIGS. 2-4.—Movement of polymorph through red cells to coccal aggregate.



FIGS. 5-6.—Polymorph applied to cocci.



FIGS. 7-8.—Beginning and extension of fissure on right side of coccal aggregate.



FIGS. 9-10.—Separation of " raft " from main coccal mass.

FIGS. 2-10.—Initial magnification ×230 ; enlarged to ×700.

## *Summary*

An apparatus and methods are described for phase-contrast cinematography of human polymorphonuclear leucocytes. In studies of phagocytosis by the methods described, it was observed that a polymorph could detach heat-killed and fixed staphylococci from the surface of a siliconed cover-slip. This phenomenon is illustrated.

Thanks are due to Mr J. Haynes for technical assistance and to the Department of Clinical Photography, Westminster Medical School, for the enlargements. The cinemicrographic apparatus was designed and made by Mr P. Cliffe, the timing device by Dr Ross Robinson.

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# THE DEMONSTRATION OF BACTERIAL CAPSULES AND SLIME

J. P. Duguid

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(Plates CXIV-CXVIII)

Extracellular material of gelatinous or gummy consistence is formed by many bacteria, particularly those giving mucoid growths. This material may remain firmly adherent as a discrete covering layer on each cell, that is as capsules, also called slime-layers or slimy envelopes, or it may part freely from the cells as loose slime, also called free slime, gum or zoögloea. Loose slime remains as a confluent matrix around the bacteria in colonies on solid medium, but disperses from the bacteria in liquid medium. Typical capsule-forming strains of bacteria show capsules on most or all of the cells in their cultures; such strains often produce much loose slime in addition to the capsules. Other strains, best designated slime-forming, produce an abundance of loose slime, but show capsules on few or none of the cells in their cultures.

The term slime-layer, or slimy envelope, has been used by some authors as synonymous with capsule, and by others as distinguishing a soft, imperfect kind of capsule which does not have the regular shape and well-defined edge of the typical capsule (Rowland, 1914; Etinger-Tulczynska, 1932-33; Knaysi, 1946; Klieneberger-Nobel, 1948). Because it has been given these diverse meanings and because it is liable to be confused with loose slime, the term slime-layer will be avoided. The term capsule will be used for any microscopically demonstrable covering layer external to the cell-wall which is firm enough to remain adherent when the cells are suspended in water. The term slime will be used exclusively to mean loose slime.

Many different methods have been suggested for microscopic demonstration of capsules and slime, but as yet none seems to inspire general confidence. Most methods are liable to occasional failure in demonstrating capsules, some falsely give the appearance of capsules around non-capsulated bacteria, and most fail altogether to reveal loose slime. The identity and equivalence of the surface structures exhibited by some of the staining methods are uncertain; their elucidation is particularly desirable in view of the great importance now attached to these surface structures in many fields of study, as in the case of bacterial virulence, variation, antigenicity and phage-adsorption (see Miles and Pirie, 1949). Because the wide diversity of methods at present used for capsules and slime makes the published results very difficult to compare and evaluate, the general adoption of one agreed method of choice would be advantageous.

For these reasons, a comparative trial of the main demonstration methods was undertaken. The wet-film India-ink method emerged as the most informative and reliable of the available methods; its general adoption is proposed, and its use in an investigation of 40 strains of *Bacterium aerogenes* and 55 strains of *Bacterium coli* is reported.

### *Strains and cultural methods*

Tests were made with a variety of strains and species, in particular with capsulated type-3 and type-19 pneumococci recently isolated from sputum, and with various non-capsulated, capsulated and slime-forming strains of *Bact. aerogenes* and *Bact. coli*. The *aerogenes* and *coli* organisms were isolated from fæces and other sources in this laboratory (A strains) or obtained from the National Collection of Type Cultures (N.C.T.C. strains); they were typed by their biochemical reactions according to the scheme of Wilson (1935). The *Bact. aerogenes* collection included 37 type-I strains and 3 type-II strains (N.C.T.C. 5939, 8167 and 8173). The *Bact. coli* collection included 52 type-I strains and 3 type-II strains.

In order that capsules and slime might be most clearly apparent, cultures were made under conditions favouring their maximum development. Inclusion of sugar or other carbohydrate in the culture medium has been found essential for production of large capsules and abundant slime in pneumococci and streptococci (Buerger, 1907; Zettnow, 1907; Morison, 1940; Wood and Smith, 1949), and in Salmonella, coliform and allied bacilli (Hoogerheide, 1939; Morgan and Beckwith, 1939; Duguid, 1948). Duguid found that *Bact. aerogenes* capsules were largest in cultures on a sugar medium wherein growth was limited by deficiency of an essential nutrient, such as the nitrogen or phosphorus source, so that after satisfaction of all the energy requirements of growth, an excess of sugar still remained available for synthesis of capsular polysaccharide; he also found that the excess-sugar medium must be well buffered, since capsule formation was slight if an acid reaction was allowed to develop. Bunting, Robinow and Bunting (1949) confirmed these findings for *Bact. aerogenes* and found that *Serratia marcescens* behaved in the same way.

In the present trial, *Bact. aerogenes* and *Bact. coli* were grown on plates of an excess-sugar peptone agar which was deficient in nitrogen and buffered with phosphate. This medium contained in 100 c.c. of distilled water: maltose, 0·5 g.; peptone (Bactopeptone), 0·05 g.; phosphate (anhydrous $Na_2HPO_4$, 3 parts; $KH_2PO_4$, 1 part; $pH = 7·3$), 0·5 or 1·0 g.; NaCl, 0·2 g.; $K_2SO_4$, 0·1 g.; $MgSO_4$, 0·01 g.; $CaCl_2$, 0·001 g.; $FeSO_4$, 0·0001 g.; washed agar powder, 2·0 g. Occasional use was made of MacConkey's lactose-peptone agar, a medium which allows moderate development of capsules and slime, but not maximum development since its sugar-peptone ratio is relatively low (1 : 2). Parallel cultures were made at 35° C. for 1-7 days and at 15-20° C. for 2 weeks, the latter being included in view of the finding by Morgan and Beckwith that a low temperature of incubation is necessary for development of mucoid growth by *Bact. coli* and certain other species. Pneumococci and streptococci were cultured at 35° C. on plates of moist blood-agar containing 0·1-0·2 per cent. glucose or sucrose, as suggested by Morison for maximum development of capsules. Microscopic observations were made by oil-immersion with a 1/12-in. apochromatic objective and a green filter.

## OBSERVATIONS ON DRIED AND FIXED FILMS

Capsules and slime are difficult to demonstrate microscopically because of their poor affinity for dyes and their liability to gross shrinkage on drying and fixing. In most methods for positive staining of capsules, the dye affinity of the capsules is first increased by application of a special fixative or mordant. In relief methods, on the other hand, the capsules are left unstained or weakly stained in contrast to a strongly stained background; the background staining is achieved by mixing the bacteria with a stainable substrate or with a special background dye. Some dry-film relief methods suffer the disadvantage that non-capsulated bacteria through shrinkage leave around themselves clear retraction zones which simulate unstained capsules; Baehr and Kantor (1912) found that such pseudocapsules were produced by the methods of Boni (1900), Hamm (1907), Medalia (1911) and Rosenow (1911).

Shrinkage and distortion of capsules were found by Baehr and Kantor to be greatest in methods involving drying followed by heat fixation: methods of Welch (1892) and of Hiss (1901-05); somewhat less with drying followed by chemical fixation: methods of Wadsworth (1906) and of Medalia (1911); and least with chemical fixation of a still moist smear: methods of Buerger (1905, 1907), of Hamm (1907) and of Rosenow (1911). Small capsules which were seen by the wet-fixation methods often could not be seen by the methods allowing greater shrinkage.

*Simple staining of heat-fixed smears.* Capsules and slime are seldom recognisable in culture smears stained in the ordinary way with a basic dye after drying and fixation by flaming. In the present trial, positive staining of capsules with methyl violet was sometimes achieved in cultures made on sugar media: for type-3 pneumococcus and for *Bact. aerogenes* (as fig. 4 of Duguid); the successful smears were made by spreading a colony directly on a slide with admixture of little or no water. Capsules of bacteria in infected body fluids are more readily stained in heat-fixed smears than capsules from cultures. In smears of the heart blood of infected mice, the type-3 pneumococcus and a *Bact. aerogenes* (A 29) regularly showed small capsules positively stained; in smears of infected peritoneal exudate, the capsules were stained less strongly, often in outline only.

*Use of special suspending media.* Many methods involve preliminary suspension of the culture in a special fluid; smears are then made from this suspension and stained with a basic dye. Serum or ascitic fluid was employed by Hiss, Buerger, Hamm, and Toenniessen (1912); glycerine, egg-albumen and formalin by Boni; and serum and phenol by Jasmin (1945). Such a procedure may minimise shrinkage and may render the capsules more stainable. In some methods, as those of Boni and Jasmin, it gives a stained background with unstained capsules showing in relief.

In trials with the copper-sulphate serum method of Hiss, the results were unsatisfactory, the capsules on some occasions being quite invisible and on others positively-stained but much shrunken in size. For the relief method of Jasmin, cultures were suspended in physiological saline containing 1 per cent. (*w*/*v*) phenol and 10 per cent. (*v*/*v*) ox-serum, and smears of this were dried, fixed with methyl alcohol and stained with methyl violet. The capsules in cultures of pneumococcus and of *Bact. aerogenes* were thus readily demonstrated, but their appearances were very variable. In different parts of the same smears, capsules were (1) apparent in relief (fig. 1), (2) outline-stained, (3) positively and uniformly stained, and (4) completely invisible.

*Use of special fixatives.* Chemical fixation of smears is better than heat fixation since it causes less shrinkage and distortion of the capsules. Moreover, certain chemical fixatives, particularly the acids, appear to increase the dye-affinity of the capsules. Heat-fixed smears were treated with acetic acid by Friedländer (1885), Welch (1892) and Cooper (1925). Dried, unheated smears were fixed with formalin by Wadsworth (1906), and with Wright's stain in absolute methyl alcohol by Wherry (1905), Churchman and Emelianoff (1933), Seastone (1934) and Sokhey (1940). Fixation of moist smears was effected with bichromate and mercuric chloride by Buerger, with osmic-acid vapour by Hamm, and with acetic acid, picric acid and formalin (Bouin's solution) by Klieneberger-Nobel.

Trial was made of Klieneberger-Nobel's method A. 1 for capsules. Fig. 2 shows successful staining of the capsule in a young colony of type-3 pneumococcus, wet-fixed through agar with Bouin's solution and stained with Giemsa's stain. However, the method was not regularly successful; often the capsules were unstained and invisible in parts of the film.

*Use of tannic acid as a mordant.* Treatment with tannic acid is especially effective for increasing the stainability of capsules by basic dyes; it also renders the cell walls stainable and the cell contents unstainable. Tannic acid is used in the capsule-staining methods of Rosenow, Muir (see Mackie and McCartney, 1948), Cooper and Klieneberger-Nobel (method A. 2).

Trial was made of Muir's method on sugar-medium cultures of type-3 pneumococcus and *Bact. aerogenes*. The results were disappointing. The capsules were positively stained, but usually appeared very small and indistinct as a result of gross shrinkage. With *Bact. aerogenes*, a clear retraction space was often seen between the red-stained bacillus and the blue-stained capsular material; this space readily might have been mistaken for the capsule.

Trial was also made of Klieneberger-Nobel's method A. 2 for capsules, using as mordant a mixture of 4 parts of 20 per cent. tannic acid, 5 parts of a saturated solution of potassium alum and 1 part of saturated mercuric chloride. Variable results were obtained. Fig. 3

DEMONSTRATION OF BACTERIAL CAPSULES

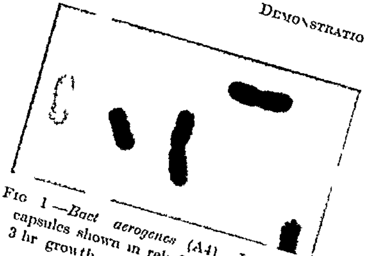

FIG 1—*Bact aerogenes* (A4) Unstained capsules shown in relief Nutrient agar 3 hr growth Jasmin's method

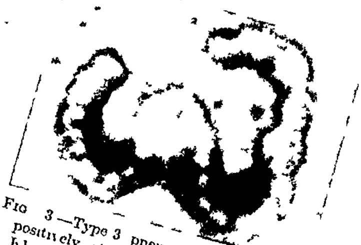

FIG 3—Type 3 pneumococcus Capsule positively stained cocci unstained Klieneberger Nobel's A 2 method

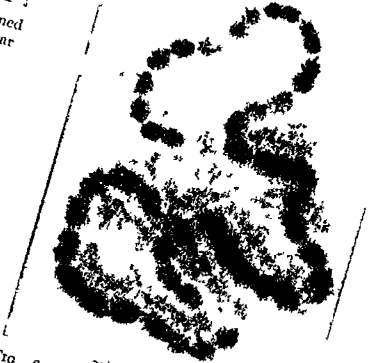

FIG 2—Type 3 pneumococcus Capsule positively stained Small 6 hr colony Klieneberger Nobel's A 1 method

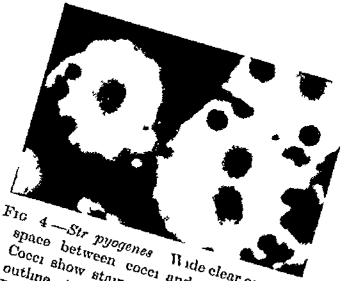

FIG 4—*Str pyogenes* Wide clear capsular space between cocci and stain deposit Cocci show stained core (? nucleus) and outline (? cell wall) Churchman and Emelianoff's method

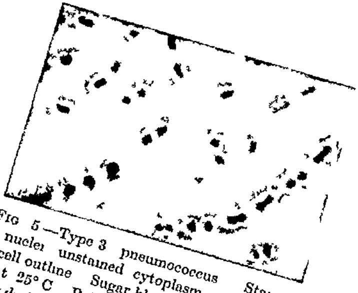

FIG 5—Type 3 pneumococcus Stained nuclei unstained cytoplasm and stained cell outline Sugar blood agar after 18 hr at 25° C Robinow's osmic vapour acid hydrolysis Giemsa method

(FIGS 1 5 ×3750)

## Demonstration of bacterial capsules

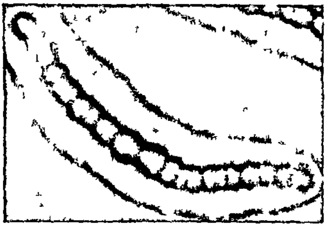

Fig 6

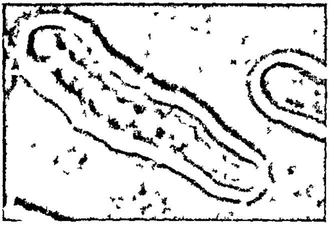

Fig. 7

Figs 6 and 7—Type 3 pneumococcus Capsules stained like background, but outlined by dye meniscus in fig 6 and clear shrinkage space in fig 7 Little slime Howie and Kirkpatrick's eosin method

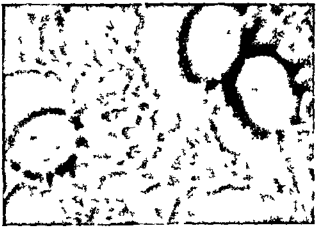

Fig 8

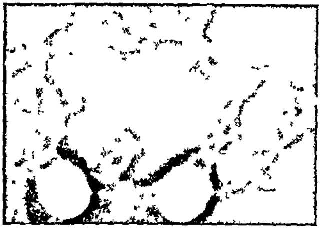

Fig 9

Figs 8 and 9—*Bact aerogenes* (A3) Capsulated bacilli with much loose slime (light fibrous deposit) After 7 days at 35° C on excess sugar peptone agar Howie and Kirkpatrick's method

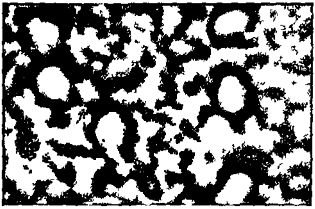

Fig 10—*Bact aerogenes* (N C T C 5936) Non capsulated bacilli with much slime After 7 days at 15 20° C. on excess sugar agar Howie and Kirkpatrick's method

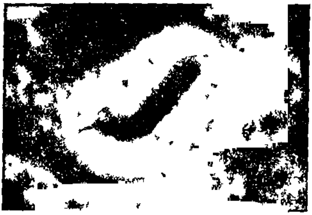

Fig 11—Type 3 pneumococcus Clear capsular space and shrunken capsule between stained cocci and background Butt, Bonynge and Joyce's dry film India ink method

(Figs 6 11: ×3750)

shows a successful preparation of type-3 pneumococcus, with large positively stained capsules. Frequently, however, the capsules were indistinguishable, being so shrunken as to simulate stained cell wall.

Klieneberger-Nobel's method B for slime involves fixation of a dried smear with Chabaud's alcoholic fixative, mordanting with tannic acid and staining with crystal violet. An objection to this method is seen in its author's statement that if the alcoholic fixative is used on capsulated bacteria, the capsules are reduced to an amorphous mass simulating slime. In the present trial, satisfactory demonstrations of loose slime were not achieved by this method.

*Method of Churchman and Emelianoff.* Churchman and Emelianoff described a method which exhibited capsules not only on known capsulated species, but also on bacteria commonly regarded as non-capsulated, such as *Streptococcus fæcalis*, R-form pneumococci, *Bacillus subtilis* and *Mycobacterium tuberculosis*. In this method smears were covered with undiluted Wright's stain and this was allowed to evaporate almost to dryness before washing. The method must be judged unsatisfactory, since it gives widely variable appearances and its exhibition of capsules on non-capsulated bacteria is almost certainly an artefact.

Trial was made of the method, using undiluted alcoholic Leishman's stain, equivalent to Wright's. Films of *Bact. aerogenes*, pneumococcus and *Streptococcus pyogenes* showed all the types of staining described by Churchman and Emelianoff. In some parts of the films the capsules were demonstrated in relief, appearing as clear unstained spaces between the darkly stained bacteria and a dark background of stain-deposit; fig. 4 shows such relief staining in a film of *Str. pyogenes*. In most parts of the films, however, the background stain was absent and the capsules were invisible. In a few fields the capsules were stained positively. Whatever the capsule staining, the bacteria themselves, like the cocci in fig. 4, showed differentiation of a stained cell outline (? cell wall), an unstained zone (cytoplasm or shrinkage space) and a stained core (nucleus or shrunken protoplast).

Churchman and Emelianoff interpreted these appearances differently; the structures described above as (1) nucleus, (2) cytoplasm, (3) cell wall and (4) capsule, they regarded as (1) stained bacterial soma, (2) unstained capsule, (3) stained capsular membrane and (4) wide "Aussenhülle", a structure supposed to lie external to the capsule. However, it seems more probable that the "Aussenhülle" is what is normally meant by "capsule", since it is found only in the species generally recognised as capsulated, while the inner structures (1-3) are seen also in non-capsulated species. The true identity of the inner structures is suggested by comparison with fig. 5, which shows the nuclear bodies, cytoplasm and cell wall of type-3 pneumococcus by the nuclear staining method of Robinow (1941-42).

*Relief methods with background dyes.* Relief demonstration of capsules in dry films may be achieved by preparing the films from a bacterial suspension which is mixed with a dye or pigment to provide a dark background. The bacteria were mixed with Congo red and serum by Fletcher (1918) and Bruce White (1947), with protargol and eosin by van Riemsdijk (1921), with broth, eosin and serum by Howie and Kirkpatrick (1934) and with nigrosin by Fleming (1941).

Nigrosin and eosin were found to behave similarly in trials with pneumococcus, *Bacillus anthracis* and *Bact. aerogenes*. The dye always infiltrated the capsule to some extent, and left the cell contents unstained. In some cases the dye concentration in the capsule remained appreciably less than in the background, so that the capsule was visible in relief as a lighter area (as in fig. 9). Usually the dye concentration in the capsule was nearly the same as in the background; then, the capsule might be indicated by a dark dye meniscus outlining its periphery (as in figs. 6 and 8), or by a clear retraction space left on its shrinkage inwards (as in fig. 7). Sometimes, however, the capsule remained invisible; especially in thick films, capsulated *Bact. aerogenes* were liable to appear indistinguishable from naked bacilli, which usually show bipolar absence of stain.

The eosin-serum method of Howie and Kirkpatrick, omitting the initial carbol-fuchsin staining, was found one of the most useful of dry-film methods (figs. 6-10). It proved reliable in demonstrating capsules, except those of small size, and it displayed the capability, not mentioned by its authors, of demonstrating clearly the presence of loose slime. Indeed, the eosin-serum method and the wet-film India-ink method were the only satisfactory methods found for the demonstration of slime; the wet-film India-ink method has the advantage of showing the slime undried and unshrivelled, but for the inexperienced observer it is more difficult than the eosin-serum method. The eosin-serum method shows slime variously as a granular, fibrous or reticular deposit appearing somewhat lighter than the general eosin background. Figs. 8 and 9 show the slime and encapsulated bacilli of a typical capsule-forming *Bact. aerogenes* strain (A 3); fig. 10 shows the slime and naked bacilli of an unusual *Bact. aerogenes* strain (N.C.T.C. 5936) which is slime-forming but non-capsulated. Fletcher (p. 103) described a similar demonstration of slime in his Congo-red preparations: he stated that in a film of non-mucoid colonies " the background of Congo-red is homogeneous, but in mucoid films it is traversed in all directions by an irregular reticulum composed of a deposit of small granules, which appears to be derived from mucilaginous matrix ".

*Dry-film India-ink methods.* Important advantages attend the use of India ink for relief demonstration of capsules; unlike the soluble background dyes, the particulate ink does not penetrate into the capsules, and it is not washed away when counterstaining of the bacterial bodies is attempted. Following Burri (1909), dry-film India-ink methods were described by Rulison (1910), Gins (1910-11), Baker (1920) and Butt, Bonynge and Joyce (1936).

## DEMONSTRATION OF BACTERIAL CAPSULES

*Figs. 12 16.—Type-3 pneumococcus. After 24 hr. at 35° C on sugar blood-agar. In wet films.*

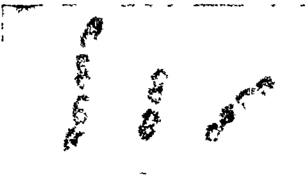

Fig. 12 —Heterologous serum with methylene blue. Capsules invisible : negative "swelling reaction".

Fig. 13 —Homologous antiserum with methylene blue. Capsule outline visible: positive "swelling reaction".

Fig. 14.—Heterologous serum with nigrosin. Capsule invisible.

Fig. 15 —Homologous antiserum with nigrosin. Capsule positively stained.

Fig. 16 —India ink. Wide capsule outside cell surface halo

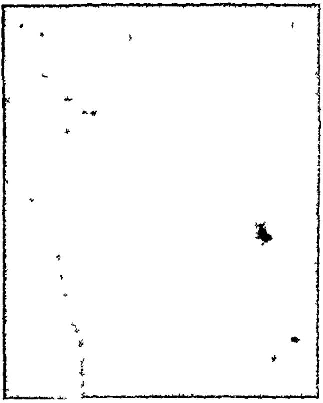

Fig. 17 —*B. megatherium.* Capsules in wet India ink film.

(Figs. 12-17 : ×3750)

By the method of Butt, Bonynge and Joyce, the culture is suspended in 6 per cent. (*w/v*) glucose solution in water and mixed with India ink; a thin film of this is dried, fixed with methyl alcohol and stained with aqueous methyl violet or other dye. In the present trial this method proved very reliable for capsules, consistently revealing those of pneumococcus, *B. anthracis* and *Bact. aerogenes*; on the other hand, it failed to demonstrate the loose slime of *Bact. aerogenes* and *Bact. coli* strains. Capsulation is indicated by the presence of a clear space between the stained bacterium and the ink background. Sometimes the capsule is faintly stained with the counterstain. When thus visible, it is seen occupying either the whole of the capsular space or only the inner part of this (as in fig. 11). In the latter case the capsule has continued to shrink inwards after setting of the ink background. As reported by Butt, Bonynge and Joyce, the capsular spaces were found to be much smaller when the initial suspension in glucose solution was omitted.

## Observations on unfixed wet films

When capsulated bacteria are examined unfixed and unstained in a film of water between slide and coverglass, the capsules normally remain invisible. In the focal plane usually chosen, the outline of the bacterial cell, whether capsulated or not, appears as a narrow dark band surrounded by a narrow bright band. These bands are refraction or diffraction halos produced by the cell surface and do not correspond to distinct surface structures. The bright halo, which may appear about 0·2 $\mu$ wide, must not be mistaken for a narrow capsule.

*Capsule-swelling reaction.* In the "specific swelling reaction" of Neufeld (1902), the outlines of capsules in a wet film are made visible by the addition of homologous antiserum. Negative and positive "swelling" reactions for type-3 pneumococcus are shown in figs. 12 and 13. The cells treated with antiserum bear capsules with refractile, clearly visible outlines (fig. 13); the cells not treated with antiserum show at their surface only the narrow diffraction halos, their capsules remaining invisible (fig. 12). The capsule is much wider than the surface diffraction halo, so that if the latter is mistaken for a narrow capsule, it will appear that the action of the antiserum is to make this swell. Almost certainly, however, the action of the antiserum is not to enlarge the capsule, but merely to render visible the outlines of a capsule which is already large. Klieneberger-Nobel in numerous tests never found antiserum to increase capsule size. This was confirmed in the present investigation; cultures of types 1, 2, 3 and 19 pneumococcus did not show appreciably larger capsules in wet films with the homologous antiserum than in wet India-ink films without antiserum (*cf.* figs. 13 and 16).

Non-specific "capsule swelling" in pneumococcus was obtained by Neufeld and Etinger-Tulczynska (1931) through treatment with certain inorganic salts, and by Jacox (1947) through treatment with various proteins at *p*H 4.

*Vital staining of capsules.* Knaysi stated that capsules are positively stained when a basic dye is added to an unfixed wet film. This was confirmed for *Bact. aerogenes* and the vitally stained capsules were illustrated in a previous paper (Duguid : figs. 11, 13, 14 and 17). The capsules of pneumococcus were found less susceptible to vital staining ; for instance, they were not stained by the methylene blue in the wet film shown in fig. 12.

*Use of background dyes in wet films.* Wet films were prepared of bacterial suspensions mixed with nigrosin or eosin to give a dark background. Usually these dyes infiltrated the whole capsule up to the edge of the cell, so that the capsule was as dark as the background and thus invisible. Fig. 14 shows type-3 pneumococcus in a wet nigrosin film ; the narrow diffraction halo is visible at the coccal surface, but the large capsule is quite invisible. In a few cases only, as with *Bact. aerogenes* grown on an excess-sugar medium, the capsule resisted entry of the nigrosin sufficiently for it to be somewhat lighter than the background and thus indistinctly visible. Loose slime could not be demonstrated in wet nigrosin or eosin films.

Addition of homologous antiserum to pneumococci in nigrosin or eosin was found to result in heavy staining of the capsules so that these appeared darker than the surrounding fluid. Fig. 15 shows type-3 pneumococcus capsules positively stained in this way with nigrosin. These dyes are thus a valuable aid for demonstration of the " swelling reaction ".

*Use of colloidal silver in wet films.* Hamm suggested the use of Collargol as preferable to India ink for relief demonstration of capsules in wet preparations. However, his illustration of *Bacterium friedländeri* in Collargol (Hamm : fig. 4) shows the capsules much less distinctly than would a well-made India-ink film. In the present trial of several silver preparations, best results were obtained with a 40 per cent. (*w*/*v*) suspension of Argyrol (silver vitellin) in water, which was just coarse enough to appear particulate through the microscope ; this showed capsules and slime in much the same way as India ink does, but it gave a lighter background and poorer contrast. The other, finer silver preparations had the fault, like nigrosin and eosin, of infiltrating the capsules and slime.

*The wet-film India-ink method.* Because of its great opacity and the inability of its particles to penetrate the polysaccharide gel, India ink provides an ideal background for the relief demonstration of capsules and slime in wet films. Subsequent to its brief suggestion by Hamm, the wet-film India-ink method was fully described by Preisz (1911), who used it for the capsule of *B. anthracis*. The method has since been used successfully to show the capsules of various species, for instance by Rowland (1914), Borin (1925) and Etinger-Tulczynska

(1932 33) Its use for demonstration of loose slime has not hitherto been described

The technique is simple and rapid, but perseverance is required for obtaining proper films The ink should be dense, homogeneous and free from large particles or clumps of particles Coarse ink may be improved by vigorous shaking with small glass beads, the author's practice is to mix the ink with a quarter volume of grade 12 (0 2 mm ) Ballotini and shake for an hour in a Mickle "Tissue disintegrator" Thin ink may be improved if concentrated somewhat by evaporation A large loopful of the undiluted ink is placed on a clean slide Into this is mixed either a small portion of colony or a small loopful of the centrifuged deposit of a liquid culture; a large number of bacteria must be added, but the ink should be diluted as little as possible A clean coverglass is placed on the drop and pressed down under a pad of blotting paper Several attempts may be necessary in order to achieve a film of the proper thickness The ink film should be about the same thickness as the capsulated bacteria, so that these are just lightly gripped between the slide and coverglass If the film is left too thick, the bacteria will drift about in the field and will be obscured by the overlying ink If the film is pressed too thin, the capsules will be flattened, distorted and perhaps disintegrated

The capsule appears as a clear light zone between the refractile cell outline and the dark ink background Typical capsules, in the absence of loose slime, are shown in figs 16, 17, 19 20 and 21, for cultured type 3 pneumococcus, *Bacillus megatherium* and *Bact aerogenes* Such capsules are fairly regular in size and shape, have clear cut margins and show little tendency to coalesce with one another They are firm in consistency, and do not disintegrate or dissolve in the ink when the wet film is kept for many hours In the centre of the capsule are seen the narrow light and dark diffraction halos which outline the surface of the bacterium itself The light halo sometimes has been mistaken for a special inner zone of the capsule, for instance, Etinger Tulczynska in describing photographs of pneumococcus and *Bact friedländeri* which resembled figs 16 and 21, gave the name "capsule" to the narrow light halo at the cell surface and the name "slime layer" to the wide capsule outside

Non capsulated bacteria, typical *Bact coli*, are shown in fig 18 These naked bacteria are not separated from the ink background by any definite clear space They show at their surface only the narrow light diffraction halo, which appears about 0 2 μ in width, hardly above the limit of resolution of the microscope In the absence of a clear zone which is definitely larger than this, bacteria should be designated "non capsulated", although the presence of a very narrow, submicroscopical capsule is never impossible Capsules of 0 25 0 5 μ in radial width are shown in fig 19, these are about the smallest which can be recognised with certainty with the ordinary microscope Naked bacteria may be difficult to see in India ink; observation is easiest when large numbers are present in a very thin film.

The wet-film India-ink method is especially suitable for the measurement of capsule size, since the capsules are not shrunken by drying. Measurement is made with a micrometer in the microscope or from a photograph taken at known magnification. The fundamental dimension is the radial thickness of the capsular layer (*e.g.* 1-1·5 $\mu$ for the cells in fig. 21); however, a more convenient measurement is the "transverse capsule diameter", including the bacterium and the capsular layers on each side of it (*e.g.* 2·5-4 $\mu$ in fig. 21). When undertaking capsule measurement it is particularly important to make films of the proper thickness; if the film is pressed too thin, the capsules are flattened and exaggerated in apparent size (*cf.* figs. 21 and 22). One must proceed by trial and error until achieving a film in which only the largest capsulated cells are gripped between slide and coverglass, while the smaller float about freely in the ink. One use of capsule measurement is in studying the factors which influence the production of capsular polysaccharide (*e.g.* Duguid). Another use may be in the distinction of strains. Measurements were made on 37 capsulated *Bact. aerogenes* strains cultured for one day at 35° C. on excess-sugar peptone agar. Most strains formed capsules of about the same size as that in fig. 21, but a few consistently formed larger or smaller capsules. The strain forming the smallest capsules is shown in fig. 19, and that forming the largest in fig. 20; the latter strain gave exceptionally stiff colonies.

When it is desired to demonstrate loose slime as well as capsules, the film must be prepared from a surface culture on solid medium. If the film is prepared from a liquid culture or a liquid suspension of a surface culture, only the capsules are seen; the loose slime is dispersed to give a uniform solution in the liquid, and as such it remains invisible. When examining for slime it is important to avoid crushing capsules by pressing the film too thin, since capsules may thus be disintegrated and fused into amorphous masses which simulate loose slime; this artefact is seen in fig. 22, a crushed film of the culture shown uncrushed in fig. 21.

Many capsule-forming bacteria produce an abundance of loose slime in addition to their capsules. All of 37 capsule-forming strains of *Bact. aerogenes* produced loose slime in cultures at 35° C. on excess-sugar peptone agar; most strains produced much slime in 24-hour cultures; 5 strains produced little slime in 24 hours (figs. 19-21), but much in 2-7 day cultures. Fig. 23 shows a typical *Bact. aerogenes* culture with large capsules and abundant slime together. Around the bacilli are seen the typical clear capsular spaces, with regular oval shape and well-defined margins. These are embedded in large irregular masses of loose slime. The slime is partly infiltrated or overlaid by the ink, so that it appears darker than the capsules, although still much lighter than the pure ink background. When the

## INDIA INK WET FILMS

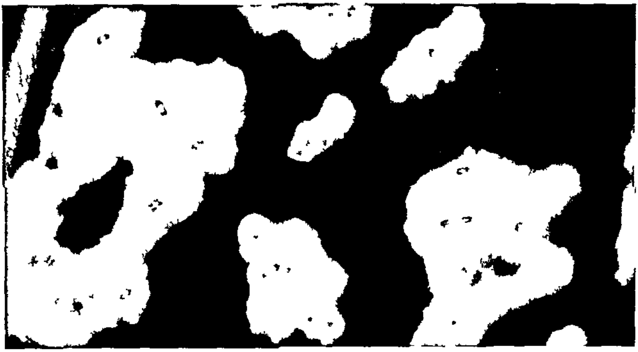

FIG 23 —*Bact aerogenes* (A3) Bacilli with large clear capsules and much loose slime, somewhat darker than capsules After 7 days at 35° C on excess sugar peptone agar

FIG 24 —*Bact aerogenes* (N C T C 5936) Non capsulated bacilli with much loose slime Some bacilli in light slime others naked in dark ink After 24 hr at 35° C on excess sugar peptone agar

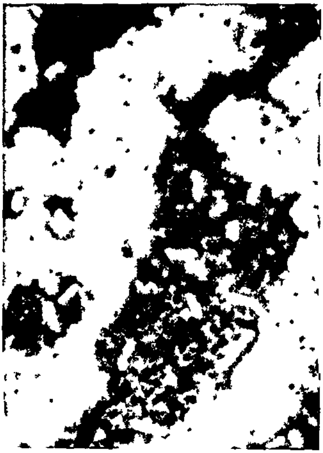

FIG 25 —*Bact coli* (A 100) Non capsulated bacilli (light rods) and much slime (large light areas) After 14 days at 15 20° C on MacConkey agar

(FIGS 23 25 × 2000)

presence of capsules in addition to slime is not obvious, it is best confirmed by suspending the surface culture in water, centrifuging and examining the deposit, this will show the capsules alone, the slime having been washed away

Many bacteria produce abundant slime in the complete or almost complete absence of capsules Purely slime forming strains of *Bact aerogenes* and *Bact coli* are shown in figs 24 and 25 Large light areas are seen which correspond to masses of loose slime not yet dispersed and dissolved in the ink Bacilli are embedded in this slime, widely and irregularly spaced (fig 24) Other bacilli, originally contained in slime which has already dissolved in the ink, are now seen lying free and naked in the ink small white rods in dark background of figs 24 and 25 Capsules are not seen in these purely slime forming strains The absence of capsules is most easily confirmed by suspending the surface culture in water, centrifuging and examining the deposit, only naked bacilli will be seen in this, the slime having been washed away

Of 40 *Bact aerogenes* strains examined in wet India ink films, 37 were typically capsule forming and 3 were purely slime forming (A 217, N C T C 5936, 8173), these three were the only strains which were motile, and the motility may well have played a part in loosening the extracellular material and preventing its retention as capsules Variation, as from a capsulated to a non capsulated form, was not observed among these *Bact aerogenes* strains during 3 years of subculturing on nutrient agar Of 52 type I *Bact coli* strains examined in cultures on the excess sugar peptone agar, 8 strains, 6 motile and 2 non-motile, were slime forming, producing abundant slime without capsules at 15 20° C, but none at 35° C, 2 strains, both non motile, were capsule-forming, producing capsules and slime together both at 15 20° C and at 35° C, and the remaining 42 strains formed neither capsules nor slime in any definitely demonstrable amount On repeated subculture a few of these eventually gave rise to slime forming variants Of 3 type-II *Bact coli* strains, none produced capsules or slime in appreciable amount Some cultures of type I *Bact coli* strains which were motile and slime forming, contained a small proportion of cells bearing capsules, such capsulated individuals were sometimes seen swimming about vigorously in the India-ink film In the main, however, capsulation and motility were not found together

## SUMMARY AND CONCLUSIONS

(1) A comparative trial was undertaken of the main methods for demonstrating bacterial capsules and slime Most methods were found liable to frequent failure in demonstrating capsules, and most methods failed altogether to demonstrate loose slime In particular, methods involving drying and fixation often produced gross shrinkage of the capsules and slime, so that these became unrecognisable

(2) Of the dry-film methods, the India-ink method of Butt, Bonynge and Joyce was found the most reliable for capsules. The eosin-serum method of Howie and Kirkpatrick was found successful for capsules, except those of small size, and in addition was one of the very few methods found capable of demonstrating loose slime.

(3) The wet-film India-ink method was preferred to all others, as the most reliable, informative and generally applicable; it proved consistently successful for capsules, even very small capsules, and for loose slime, showing these in their natural form and size without shrinkage. A description is given of this method, and of its use for measurement of capsule size.

(4) In an investigation by the wet-film India-ink method, 37 of 40 strains of *Bact. aerogenes* were found to be capsule-forming and 3 strains purely slime-forming. Of 55 *Bact. coli* strains, 2 were found to be capsule-forming, 8 slime-forming and 45 neither capsule-forming nor slime-forming to a clearly demonstrable extent.

I wish to thank Professor T. J. Mackie for his encouragement and advice, and I am indebted to Miss M. J. MacPherson and Miss M. C. Kelly for isolating and identifying the *Bact. coli* strains.

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# THE INCIDENCE AND PROBABLE SIGNIFICANCE OF PHARYNGEAL INCLUSION BODIES, WITH SPECIAL REFERENCE TO THEIR PRESENCE IN NEW-BORN INFANTS

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(Plates CXIX and CXX)

During the investigation of an outbreak of pneumonia of suspected viral origin among the infants of a maternity unit in Nottingham, eosinophil cytoplasmic inclusion bodies were found in smears from the throats not only of the infants with pneumonia but also regularly in the smears of throats of infant contacts and of infants in unaffected nurseries of the same hospital. These results were unexpected, for Adams and his colleagues (1942), although finding similar inclusion bodies in certain epidemics of neonatal pneumonia, obtained a very low incidence of the inclusions in control infants. Because of this discrepancy a careful study was made of smears from the infants in the nursery of another maternity unit in Nottingham and from the infants in a maternity hospital in Edinburgh. Smears were also made at another Edinburgh maternity hospital from infants under two days old and at the time of birth from a few infants, some of whom were delivered by Cæsarean section. Finally smears were taken from infants at the hospital in which the original epidemic of pneumonia had occurred, but about a year later, and this time smears were also taken from the throats of their mothers.

A comparison has also been carried out on the relative incidence of inclusions in the throat and in the tongue in small groups of infants and adults. Sections of tongue from a few fœtuses or infants dying within a few days of birth have also been examined.

## Material and methods

*Collection of specimens.* Swabs were taken from the back of the throat and either smears were made at once or the swabs were immersed in a small quantity of saline and after return to the laboratory smears were made from the centrifuge deposit.

*Staining methods.* Careful heat fixation was found satisfactory for the smears. Tissues were fixed in 5 per cent. formalin. Mann's methyl blue-eosin stain was used at first with fairly satisfactory results but later, after experimenting with a number of recognised stains for inclusion bodies, Lendrum's phloxin-tartrazine method (1947) was found to be the most satisfactory and was adopted as a routine. The nigrosin technique described by Broadhurst *et al.* (1936) but without counterstain gave good results and was used during part of the investigation in parallel with Lendrum's stain.

*Microscopy.* The 1/6 in. objective and a low-power ocular were found to be the most useful for routine examination of smears. The 1/12 in. objective was used mainly for checking doubtful findings and studying details of morphology.

## FINDINGS

### *Appearance of inclusion bodies*

*Smears.* Cytoplasmic inclusions are found in squamous epithelial cells in smears of the pharynx or buccal cavity. Apart from the inclusions the affected cells usually appear quite normal. The inclusions in a cell vary in number from one to over 20 and are of greatly varying size (fig. 1). Several inclusions in a cell are commoner than a single one. Fig. 2 shows a typical appearance. When smears contain few affected cells the number of inclusions in a cell appear to be on the average fewer than in smears in which inclusions are common. When inclusions are abundant it is noticeable that the distribution may be very irregular, some epithelial sheets consisting of cells all heavily affected (fig. 4) while neighbouring sheets of cells may be practically free of inclusions.

The inclusions vary in size from granules less than 1 $\mu$ in diameter to bodies of about 6 $\mu$ diameter, but inclusions larger than 3 or 4 $\mu$ are uncommon. The inclusions are usually round but, particularly when large, they may be oval or elongated. They may be found in any part of the cell, but the larger ones tend to be near the nucleus, which they may indent (figs. 1 and 5). A characteristic appearance is that of two inclusions of moderate size on opposite sides of the nucleus (fig. 3). It has not usually been possible to distinguish any structure in the inclusions, but a surrounding halo is sometimes seen, or a clear space in the centre of the inclusion.

The inclusions are moderately eosinophil and fuchsinophil and stain with hæmatoxylin, 1 per cent. nigrosin (technique of Broadhurst *et al.*, 1936), methylene blue, and Giemsa's stain. They are Gram-negative, give a negative Feulgen reaction and do not stain by the periodic acid-Schiff technique or with Sudan black B. The stain giving the most reliable results has been Lendrum's phloxin-tartrazine. By this method the inclusions usually stain a deep red, but there may be considerable variation in the intensity of staining. With Broadhurst's nigrosin method the inclusions stain all shades from light grey to deep black. When incubated with trypsin or pepsin the inclusions disintegrate and become unstainable.

PHARYNGEAL INCLUSION BODIES

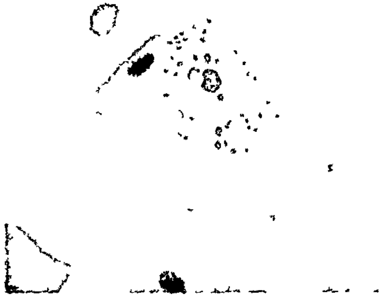
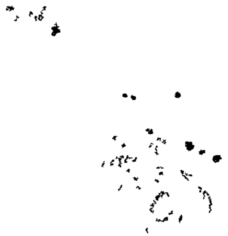


Fig 1—Pharyngeal smear from eleven day old infant showing an epithelial cell with numerous inclusions, including a large one indenting the nucleus *Acid* hæmalum phloxin tartrazine ×550

Fig 2—Pharyngeal smear from new born infant showing characteristic appearance of inclusions in three epithelial cells Mann's methyl blue eosin stain ×550



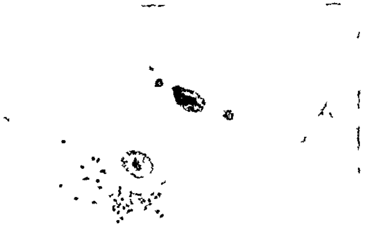


Fig 3—Pharyngeal smear from ten day old infant showing cell with two inclusions with bipolar arrangement Acid hæmalum phloxin tartrazine ×550

With some experience the inclusions can also be seen in wet unstained preparations as rounded bodies resembling the cell nucleus in refractivity and density By pressure on the coverslip it is sometimes possible to roll an inclusion

*Sections* Sections of tongues, stained by Lendrum's phloxin tartrazine technique, have been examined from stillborn fœtuses of 16 weeks, 28 weeks and 30 weeks and from one stillborn infant a few weeks premature Tongue tissues from two infants dying a few days after birth have also been examined In the 16 week fœtus no inclusions could be seen in the lingual epithelium, but in the other fœtuses and infants some inclusions, similar in appearance to those found in smears, were present in some of the most superficial epithelial cells The inclusions were least numerous in the 28 week and 30 week fœtuses Fig 6 shows the characteristic appearances and situations of the inclusions

## *Incidence of inclusions*

Altogether over 100 healthy babies have been swabbed Samples were taken in two hospitals in Nottingham and two in Edinburgh The ages of these infants varied from the time of birth to 11 days Nine infants were swabbed at birth, 6 of them following Cæsarean section In all the infants, including those delivered by Cæsarean section, inclusion bodies have been found in varying proportions of the pharyngeal epithelial cells An attempt was made to determine the percentage of cells affected in each smear, but it was not possible to obtain accurate figures owing to the irregular distribution of the

TABLE I

*Incidence of cells containing inclusion bodies in four groups of new born infants*

| Hospital | Total infants | No of infants in each incidence group * | | | | |
|---|---|---|---|---|---|---|
| | | 0 | ± | + | ++ | +++ |
| *Pharyngeal smears* | | | | | | |
| A (Nottingham) | 18 | 0 | 2 | 6 | 3 | 7 |
| B (Nottingham) | 21 | 0 | 5 | 4 | 5 | 7 |
| C (Edinburgh) | 16 | 0 | 3 | 3 | 5 | 5 |
| Total | 55 | 0 | 10<br>18 2<br>(per cent ) | 13<br>23 6<br>(per cent ) | 13<br>23 6<br>(per cent ) | 19<br>34 5<br>(per cent ) |
| *Tongue smears* | | | | | | |
| A (Nottingham) | 27 | 0 | 1<br>3 7<br>(per cent ) | 1<br>3 7<br>(per cent ) | 8<br>29 6<br>(per cent ) | 17<br>63 0<br>(per cent ) |

* 0 = <1 per cent of cells affected ± = 1 5 per cent + = 6 10 per cent ++ = 11 20 per cent +++ = over 20 per cent

affected cells and to the frequent occurrence of the cells in sheets or clumps. However, the children were grouped according to the percentage of cells affected—under 1 per cent., 1-5 per cent., 6-10 per cent., 11-20 per cent. and over 20 per cent. of the epithelial cells affected. The results gave a fairly good indication of the variation in incidence from infant to infant. Table I shows that there is no great variation in the incidence of inclusions in children in different hospitals but emphasises the wide variations that may occur from infant to infant. It also shows the higher incidence of inclusions in tongue smears. In 8 infants smears were taken on two or more occasions; it was found that each infant tended to maintain about the same incidence of affected cells over the period of observation, but some exceptions occurred.

To determine whether there was any relation between the presence of inclusion bodies in the mother and her infant, a group of mothers and babies were swabbed and table II shows the results. It will be seen that inclusions were much scarcer in the mother and there is no clear evidence of relationship between the number of affected cells in mother and infant.

TABLE II

*Incidence of pharyngeal epithelial cells containing inclusion bodies in a group of infants and their mothers*

| Infant | Age (days) | Incidence of inclusions in infant * | Incidence of inclusions in mother * |
|---|---|---|---|
| 1 | 9 | ± | ± |
| 2 | 9 | ± | ± |
| 3 | 10 | + | + |
| 4 | 11 | ++ | ++ |
| 5 | 1½ | ++ | ++ |
| 6 | 8/24 | + | ± |
| 7 | 7 | + | ± |
| 8 | 8 | + | ± |
| 9*a* | 8 | +++ | ++ |
| 9*b* | 8 | ++ | ++ |
| 10 | 8 | ++ | ± |
| 11 | 5 | ++ | ± |
| 12 | 3 | +++ | ± |
| 13 | 6 | +++ | ± |
| 14 | 8 | +++ | ± |
| 15 | 3 | +++ | ± |
| 16 | 6 | +++ | ± |
| 17 | 6 | +++ | 0 |

* 0 = 0 in 300 cells; ± = under 5 per cent.; + = 6-10 per cent.; ++ = 11-20 per cent.; +++ = over 20 per cent.

Table III shows the relative incidence of inclusions in throat and tongue of groups of infants. There is a distinctly higher incidence in the tongue than the throat. Examinations made in a group of 12 adults gave similar findings, the mean percentage of affected cells in smears from the throat being 5·3 and from the tongue 13·0.

# PHARYNGEAL INCLUSION BODIES

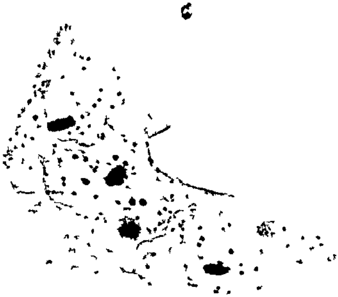


Fig 4 —Pharyngeal smear from two day old infant showing numerous inclusions throughout a clump of epithelial cells Acid hæmalum phloxin tartrazine ×550

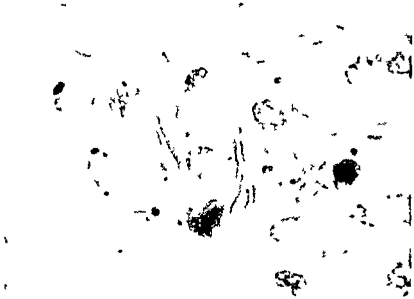


Fig 5 —Pharyngeal smear from four day old infant showing inclusions in a number of cells including three with the inclusion close to the nucleus The nucleus is indented in one of these cells Acid hæmalum phloxin tartrazine ×550

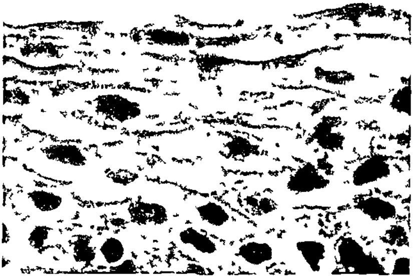


Fig 6 —Section of tongue of new born infant showing cytoplasmic inclusions in the surface layer of cells Iron hæmatoxylin phloxin tartrazine ×1000

## DISCUSSION

Cytoplasmic inclusion bodies in the pharyngeal epithelium from cases of neonatal pneumonitis have been described by Adams and his colleagues (1942, 1948*a*, 1948*b*) and in adults, often associated with mild upper respiratory tract infection, by Broadhurst and her co-workers (1936, 1943) and by de Berardinis (1948). These workers have been inclined to regard the inclusions as evidence of virus infection and Broadhurst and her colleagues (1938) claim to have infected monkeys, chick embryos and tissue cultures with material (including filtrates) from " carriers ".

TABLE III

*Incidence of cells containing inclusion bodies in smears from mouth and throat in 15 new-born infants*

| Infant | Affected cells—throat (per cent ) | Affected cells—tongue (per cent ) |
|---|---|---|
| 1 | 5 | 18 |
| 2 | 8 | 24 |
| 3 | 9 | 17 |
| 4 | 9 | 34 |
| 5 | 10 | 9 |
| 6 | 10 | 21 |
| 7 | 10 | 25 |
| 8 | 11 | 18 |
| 9 | 12 | 41 |
| 10 | 13 | 4 |
| 11 | 15 | 20 |
| 12 | 15 | 31 |
| 13 | 18 | 16 |
| 14 | 20 | 51 |
| 15 | 28 | 31 |
| | Mean = 12 9 | 24 0 |

100 cells examined in each smear

The inclusions described by these workers are indistinguishable in size, shape and staining characteristics from those found in the present investigation and it seems highly probable that they have the same origin. They have, however, been found not only in all infants examined but also in infants at birth delivered either by Cæsarean section or naturally, and in stillborn infants whether premature or full term. They have also been found in 31 of 32 adults examined. Sections show these inclusions in the most superficial epithelial layers of the mucous membrane, usually with a patchy distribution. These results indicate that a viral origin for the inclusions is very improbable, for this would imply a universal prenatal infection of infants, limited to the most superficial layers of stratified epithelium. Moreover a satisfactory alternative explanation is available.

The cells of the *stratum granulosum* of the skin contain large numbers of granules (keratohyalin) and a few are also found in the most superficial cells of the Malpighian layer. These granules stain in the same way as the inclusion bodies under discussion. It is probably significant that Mallory (1938) refers to a nigrosin technique for staining these granules and nigrosin has also been found useful for the pharyngeal inclusions. In the mouth and throat the *stratum corneum* is normally absent and a rudimentary *stratum granulosum* lies close to the surface. It seems probable that the cells found in smears to contain inclusions come from this layer.

Ludford (1924-25) claimed that in the Malpighian layer of mouse skin the nucleoli were extruded into the cytoplasm where they swelled and fragmented to form keratohyalin granules. The earliest change described by Ludford was the appearance of one or two nucleolar extrusions in the cytoplasm close to the nucleus, sometimes conspicuously indenting the nucleus. These appearances correspond very closely to those found in tongue smears. The nucleoli show the same staining characters as the inclusions. It would seem reasonable to explain the inclusions as an early stage in the formation of keratohyalin possibly resulting from nucleolar extrusion.

With regard to the reported finding of inclusions in chick embryos and tissue cultures inoculated with throat material it may be noted that Goldsworthy and Moppett (1935) and D'Aunoy and Evans (1937) have described inclusions in chorio-allantoic membranes not inoculated with viruses. Drew (1922) found that embryo skin cultures after 5-6 days began to show signs of keratinisation and Lewis (1922) described nucleolar extrusions in old tissue cultures of endothelial cells. Fischmann and Russell (1940) also described intra-nuclear inclusions developing in tissue cultures of human fœtal leptomeninges and in fibroblast cultures from human fœtal lungs. In some preliminary tissue cultures carried out in the present work large spherical inclusions, one or two to a cell, were found in some of the cells of rabbit corneal epithelium after 7 days' tissue culture. These various reports suggest therefore that inclusions such as those described by Broadhurst in tissue cultures are to be found in normal cells.

## SUMMARY

1. Cytoplasmic inclusion bodies have been found in the squamous epithelium of the mouth and throat of all new-born infants examined (over 100). They were also found in premature stillbirths and in live infants at the time of birth delivered naturally or by Cæsarean section.

2. Histological examination showed that the inclusions were confined to cells in the most superficial layers of the mucous membrane.

3. The inclusions were found in 31 out of 32 adults, but in much smaller numbers.

4. There is no relation between the numbers of inclusions found in mother and new-born infant.

5. The inclusions are more numerous in tongue smears than in throat smears and in adults it may be difficult to find them in the throat.

6. These results differ from those of previous workers who found inclusions in a limited number of infants or adults and related their incidence to the presence of neonatal pneumonitis or, in adults, to mild upper respiratory tract infection.

7. Reasons are given for suggesting that the inclusions are derived from nucleolar extrusions produced at an early stage in the natural ageing of the epithelial cell and may be regarded as an early stage in keratohyalin production.

I wish to express my indebtedness to Dr A. P. M. Page for drawing my attention to the pneumonia epidemic that initiated this investigation and to thank Dr J. B. Cochrane, Professor Richard Ellis and Dr Mary I. Gibson for allowing access to clinical material, and to Dr Agnes McGregor for sending autopsy material. Thanks are also due to Professor T. J. Mackie for providing laboratory facilities during part of this investigation and to Dr R. G. Mitchell for obtaining smears from babies at Cæsarean section. I also wish to express my appreciation of the skill of Mr T. C. Dodds of Edinburgh University Pathology Department, who is responsible for the photographs, with the exception of fig. 6 for which I have to thank Mr P. H. Johnson of Nottingham.

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576 . 809 . 72 : 576 . 851 . 46 (*H. influenzæ*)

# THE OCCURRENCE OF TWO DISTINCT CAPSULAR ANTIGENS IN *H. INFLUENZÆ* TYPE e STRAINS

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In November-December 1948 six capsulated strains of *Hæmophilus influenzæ*, requiring both the X and V factors, growing as mucoid colonies and showing iridescence on Levinthal agar plates, were isolated from pernasal swabs of children suspected of having whooping cough, and found to be untypable with the six specific type antisera a-f prepared in this department in 1942. All six type antisera were re-tested with the stock strains of these six *H. influenzæ* types and found to give a specific precipito-agglutination with the homologous strain using the slide and tube agglutination tests as carried out in this department (Allison, Gordon and Zinnemann, 1943), as well as producing a specific " capsule swelling " or Neufeld effect.

It was thought at first that a hitherto unknown type had been isolated. However, when the strains were tested with a set of antisera made in the Commonwealth Serum Laboratories, Melbourne, Australia, it was found that the Australian type e antiserum agglutinated these strains and our stock type e strain. This discrepancy seemed worthy of investigation as with our own type e antiserum we had been able to identify one other type e strain from a similar case during the same period.

## Experimental

Accordingly, two rabbits were immunised with two of the freshly isolated strains (He. and Ib.) and after titres of 1 in 320 and 1 in 160 respectively were attained the rabbits were bled and the sera pooled. All six strains as well as the type e stock strain gave a precipito-agglutination with this new antiserum, labelled hereafter HI; our original type e antiserum will be referred to as e 1942. In the tube agglutination tests these same seven strains gave a precipito-agglutination in serum dilutions of 1 in 80, with marked disc formation in the 1 in 10 and 1 in 20 dilutions. Table I shows the results of the cross Neufeld tests.

The cross Neufeld reactions suggested that the six newly isolated strains possessed an antigen component, probably on the surface, which was absent in the stock type e strain, and that the corresponding antibody was absent in our type e 1942 antiserum. To confirm this supposition the e 1942 antiserum was absorbed with the Ib. strain of the freshly isolated group and the HI antiserum absorbed with our stock type e *H. influenzæ* strain. The absorbed antisera were then tested against five of the freshly isolated strains—one of the six strains having meanwhile passed into the rough phase—against four similar strains isolated during the course of the investigation, and against the stock type e strain.

TABLE I

*Cross Neufeld tests with six freshly isolated and stock type e* H. influenzæ *strains*

| Strain | e 1942 antiserum | Melbourne type e antiserum | HI antiserum |
|---|---|---|---|
| Ba. . . . . | 0 | 2+ | 3+ |
| Ga. . . . . | 0 | 1+ | 3+ |
| He. . . . . | 0 | 2+ | 3+ |
| Ib. . . . . | 0 | 1+ | 3+ |
| Ma. . . . . | 0 | 2+ | 3+ |
| Me. . . . . | 0 | 2+ | 3+ |
| Stock e . . . . | 3+ | 2+ | 3+ |

3+ = capsular diameter twice bacterial width. 2+ = capsular diameter equal to bacterial width. 1+ = capsule visible but diameter less than bacterial width. 0 = no capsule visible.

The e 1942 antiserum was completely absorbed by the Ib. strain and gave after absorption no precipito-agglutinations with any strain tested in serum dilutions greater than 1 in 10. The HI antiserum absorbed with the type e stock strain gave well marked though reduced precipito-agglutination titres with the freshly isolated strains, but not with the stock type e strain, as shown in table II.

TABLE II

*Precipito-agglutination titres of HI antiserum absorbed with stock type e strain*

| Strain | Dilution of absorbed HI antiserum | | | | | |
|---|---|---|---|---|---|---|
| | 1 : 10 | 1 : 20 | 1 : 40 | 1 : 80 | 1 : 160 | 1 : 320 |
| Ba. . . . . | 3+ | 3+ | (+) | 0 | 0 | 0 |
| Ga. . . . . | 3+ | 3+ | 2+ | 0 | 0 | 0 |
| Ib. . . . . | 1+ | 1+ | 1+ | 1+ | 1+ | 0 |
| Ma. . . . . | 1+ | 2+ | 2+ | 0 | 0 | 0 |
| Me. . . . . | 2+ | 2+ | 1+ | 1+ | 0 | 0 |
| Jo. . . . . | 2+ | 3+ | 2+ | 0 | 0 | 0 |
| 5892 . . . . | 3+ | 3+ | 0 | 0 | 0 | 0 |
| Bl. . . . . | 3+ | 3+ | 1+ | 0 | 0 | 0 |
| 10168 . . . . | 3+ | 3+ | 1+ | 0 | 0 | 0 |
| Stock e . . . | 0 | 0 | 0 | 0 | 0 | 0 |

3+ = disc formation at bottom of tube. 2+ = large floccules. 1+ = small floccules. (+) = fine granules. 0 = no agglutination.

The HI antiserum absorbed with the stock type e strain gave "capsule swelling" 1+ with the freshly isolated group of strains but not with the stock type e strain. The e 1942 antiserum absorbed with the Ib. strain gave no visible Neufeld reaction with either the freshly isolated group of strains or the stock type e strain.

The question then arose whether a somatic antigen was perhaps involved in the phenomenon observed. Fortunately an antiserum against the He. strain in the capsulated phase had been prepared before the strain passed into the non-capsulated phase. This serum also constituted one of the two components in the pooled HI antiserum. This He. antiserum when absorbed with the non-capsulated He. strain continued to give a precipito-agglutination to titre with the freshly isolated strains. When further absorbed with type e stock strain this twice absorbed serum was found not to differ from the HI antiserum absorbed with type e stock strain. There can be little doubt that the freshly isolated strains possessed a capsular antigen component additional to the one present in our stock strain.

## Discussion

A similar observation had been made earlier by one of us, when a mucoid *H. influenzæ* strain isolated from a bronchial secretion could not be typed at first, but gave a very well-defined and specific precipito-agglutination with our e 1942 antiserum after subculture on chocolate agar for some time. As the significance of this isolated observation was not realised at the time the strain was reported as type e without giving any further details (Allison *et al.*). This observation was made when the e 1942 antiserum had been newly prepared, and indicates that the absence of the additional antibody in the e 1942 antiserum in the year 1950-51 is not due to a slow deterioration of the serum during the course of years. It seems probable, though, that this strain lost the additional antigen during prolonged subculture on laboratory media and that this change eventually enabled us to determine the strain as type e.

There are some isolated references in the literature to untypable strains (Straker, 1945; Smith, Wilson and Hodes, 1946; Weinstein, 1946). Neither Straker nor Weinstein appears to have had a complete set of type antisera. It is clear, however, that Smith *et al.* did have a complete set; one may speculate whether their strain was a non-capsulated one or whether our observations explain their failure to type it.

The ten freshly isolated type e strains in which the additional antigen was observed grew as very mucoid colonies and with the exception of one strain have retained this characteristic for more than two years. During this time the precaution was taken to subculture on thick chocolate agar plates and in defibrinated rabbit's blood. The exception referred to was strain He., which passed into the rough phase before completion of this investigation. It has not been possible to obtain reversion of this strain into the smooth phase by twelve consecutive subcultures in 10 per cent. anti-R-serum-Levinthal-broth. The stock *H. influenzæ* type e strain corresponding to the e 1942 antiserum has varied between a smooth, not very mucoid and an excessively mucoid phase and vice-versa. In both phases precipito-agglutination and " capsule swelling " were obtained with the e 1942 antiserum. This behaviour would appear to rule out the loss of an antigen component peculiar to the mucoid phase. The M→S⇌R variation postulated by Chandler *et al.* (1939) obviously does not play any part in the phenomenon observed.

It remains to label the two distinct capsular antigens. As the antigen present in the freshly isolated strains is probably the more superficial one we propose to designate this the " *H. influenzæ* type e antigen 1 ". For the antigen observed in the stock strain and apparently also present in a deeper layer of the freshly isolated strains we suggest the name " *H. influenzæ* type e antigen 2 ". Thus strains may be found which possess both the e 1 and e 2 capsular antigens (our freshly isolated strains) and others which possess only the e 2 capsular antigen (our stock type e strain).

The type e stock strain and the strain Ib. have been deposited with the National Collection of Type Cultures, and have been given the numbers N.C.T.C. 8455 and N.C.T.C. 8456 respectively.

## SUMMARY

Two distinct capsular *H. influenzæ* antigen components have been demonstrated, one (e 2) in a stock type e strain, and the same, together with an additional and more superficial antigen (e 1), in some freshly isolated strains of type e *H. influenzæ*.

We should like to express our gratitude to Dr E. A. North for the gift of specific *H. influenzæ* antisera types a-f prepared in the Commonwealth Serum Laboratories, Melbourne, Australia. Our thanks are also due to Mrs B. Dawson and Dr J. W. Czekalowski for the strains isolated from whooping-cough suspects.

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616 . 61—056 . 55—092 . 9 : 547 . 821 (pyridoxin)

# RENAL LESIONS IN PYRIDOXIN DEFICIENT RATS

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(Plates CXXI and CXXII)

During studies on antibody production in pyridoxin deficient rats (Agnew and Cook, 1948 49), severe hæmaturia was observed in several animals given a pyridoxin deficient diet but not in corresponding litter mates fed the complete diet either in reduced amount (paired weighed) or *ad libitum* A detailed study (Agnew, 1949) was made of the incidence, duration and severity of the hæmaturia, since only Birch (1938) and Cerecedo and Foy (1944) have commented on this subject This paper describes the renal lesions which were observed in most of the rats that had developed hæmaturia

## Methods

The animals used were (1) hooded Lister rats (Rowett Institute strain), and (2) albino rats obtained from the Agricultural Research Council Field Station, Compton, these were originally bred by Glaxo Laboratories Ltd from brother sister matings of Wistar rats Each experiment in the present series involved the use of several trios of litter mate weanling rats of the same sex and approximately the same weight (table I), each trio of animals being arranged thus

rat no 1 (normal control) received unlimited amounts of the experimental diet with all vitamin supplements,
rat no 2 (pyridoxin deficient) received the diet *ad lib* with all vitamin supplements except pyridoxin,
rat no 3 (inanition control) was paired weighed with rat no 2 but was given pyridoxin *i e* it received the amount of diet required to keep it equal in weight to rat no 2

The synthetic diet contained vitamin free casein, sucrose, fat, salts and purified vitamins (see Agnew and Cook) The animals were housed in individual cages with wide wire screened bases, they had unlimited access to water and were weighed daily, except on Sundays

*Experiment* 1 *short term deficiency* (53 *days*) Seven trios of litter mate hooded rats were used, four of males and three of females These rats had been used in studies on antibody production (Agnew and Cook) and each had received an intraperitoneal injection of 2 ml of a formolised suspension (" H " antigen) of *Bact typhosum* (strain Kasauli) on the 43rd day of the experiment

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*Experiments* 2-5 : *long-term deficiency* (79-175 *days*). Seventeen trios of litter-mate hooded rats were arranged in dietary groups as shown in table I. The reasons for using different fats in experiments 3 and 4 are indicated on p. 702 of this communication and more fully discussed by Agnew.

TABLE I

*Plan of experiments*

| Experiment no.* | Strain of rat | Trios examined | | | Fat used † (5 per cent. of synthetic diet) |
|---|---|---|---|---|---|
| | | No. | Sex | | |
| | | | Male | Female | |
| 1 | Lister, hooded | 7 | 4 | 3 | Margarine |
| 2 | ,, | 5 | 2 | 3 | ,, |
| 3 | ,, | 6 | 3 | 3 | Lard |
| 4 | ,, | 6 | 3 | 3 | Margarine plus linoleic *acid* (*0·75 per cent.*) |
| 5 | Wistar, albino | 6 | 3 | 3 | Margarine |

* Experiment 1 lasted 53 days *i.e.* short-term pyridoxin deficiency ; experiments 2-5 lasted from 79-175 days *i.e.* long-term pyridoxin deficiency.

† Different fats were used in experiments 3 and 4 for reasons stated in the text and fully discussed in the paper by Agnew.

After the rats died, or were killed with coal gas, the heart and kidneys were weighed, fixed in 10 per cent. formol-saline or Helly's fluid, and paraffin sections, 4-6 $\mu$ in thickness, were stained with Harris's hæmatoxylin and eosin. Several kidney sections were also stained by Masson's trichrome method, Weigert's fibrin stain, von Kossa's silver nitrate method for calcium, and the periodic acid-Schiff method.

*Detection of hæmaturia.* A simple, rapid method devised for the examination of urines for red blood cells has already been fully described (Agnew, 1949, p. 219). Filter paper was placed below the cage where urine would fall on it. The paper was examined for blood by the naked eye and by the benzidine reaction. If this was positive a sample of urine was collected for microscopic examination. A negative benzidine reaction was regarded as proof that the urine did not contain blood.

## RESULTS

*Experiment* 1 (*short-term deficiency*). Hæmaturia was observed in 4 of the 7 pyridoxin-deficient rats shortly before they and the corresponding controls were killed on the 53rd day of the experiment. Hæmaturia was not observed in any of the control rats. Macroscopically, the kidneys of the pyridoxin-deficient rats appeared normal. Microscopically, however, abundant deposits of amorphous, faintly eosinophilic material were present in the subcapsular spaces of 5 of the 7 pyridoxin-deficient rats. From 25-50 per cent. of the subcapsular spaces were involved in 2 of these rats, and about 10 per cent. in the remaining 3 rats. Only in one of the 7 inanition control rats were comparable deposits seen, about 10 per cent. of subcapsular spaces being affected ; in this animal several red cells were present in a few of the tubules of the renal papilla. Reduced intake of food may render a rat more than normally sensitive to lack of pyridoxin (Agnew, 1949, p 230), and it is possible that pyridoxin deficiency may have been brought about in some unknown way in this animal Hæmaturia was not observed in this animal but it could have been missed if it began only a few hours before death

Fig 1 suggests that the subcapsular material is derived from blood that has leaked through the glomerulus Red cells were not, however, observed in the subcapsular spaces, and examination of one or two sections with Weigert's fibrin stain indicated that the deposits were not fibrin The deposits were not birefringent The material seems to pass into the proximal tubule (fig 1) and thence through the entire nephron In this short term experiment, these subcapsular deposits were the only obvious renal lesions, apart from the red cells in the tubules of the one inanition control already mentioned Thus, the earliest lesion in a pyridoxin deficient rat with hæmaturia appears to be one affecting the glomerular filter, and this lesion may be responsible for the amorphous material found in the subcapsular spaces, and the red cells found in the urine

Four of the 5 pyridoxin deficient rats whose kidneys showed subcapsular deposits developed hæmaturia The hæmaturias observed, however, were of recent origin and hæmaturia occurring undetected a few hours before death may have been the explanation of this apparent discrepancy

*Experiments 2 5 (long term deficiency)* Macroscopically, scarring and pitting were observed in the kidneys of several of the pyridoxin-deficient hooded rats that had protracted hæmaturia (table II, experiments 2 4) These lesions were not seen in the kidneys of the pyridoxin deficient albino rats (table II, experiment 5) Deposition of yellowish cretaceous material at the tip of the renal papilla was

TABLE II

*Incidence and duration of hæmaturia and of naked eye renal lesions in pyridoxin deficient rats of experiments 2 5*

| Expt no | Strain of rat | Incidence of hæmaturia | | Duration of hæmaturia (days) | | Incidence of naked eye renal lesions | |
|---|---|---|---|---|---|---|---|
| | | No with hæmaturia | No of pyridoxin deficient rats | Range | Arithmetic mean | No with lesions | No of pyridoxin deficient rats |
| 2 | Lister, hooded | 5 | 5 | 7 147 | 92 | 4 | 5 |
| 3 | ,, | 6 | 6 | 71 115 | 98 | 1 | 6 |
| 4 | , | 6 | 6 | 2 61 | 35 | 4 | 6 |
| 5 | Wistar, albino | 0 | 6 | | | 0 | 6 |
| Totals for Lister hooded rats | | 17<br>100 per cent | 17 | | | 9<br>53 per cent | 17 |

noted in a few instances, and this was verified histologically. Hydronephrosis was noted in one rat. Possibly the hydronephrosis resulted from partial or intermittent obstruction of the ureter by a piece of this material. The affected kidney was large and pale, and clear fluid escaped from the greatly dilated pelvis when the kidney was sliced. Scarring was not observed in any of the control rats except in 2 of the *ad lib.* control rats that had received a linoleic acid supplement (table I, experiment 4). Both of these rats and 3 others given the linoleic acid supplement developed severe hæmaturia and renal lesions similar to those described below. It is likely that the supplement of crude linoleic acid had a toxic effect on the animals, or adversely affected the pyridoxin content of the diet. These possibilities are more fully discussed by Agnew.

Microscopically, examination of the scarred and pitted kidneys confirmed the suspicion that the naked-eye appearance was the result of fibrosis. In the kidneys without macroscopic lesions, however, areas of early fibrosis were seen. More severe fibrosis, with tubular dilatation, is shown in fig. 2. Areas of tubular dilatation unassociated with obvious fibrosis were present in a few kidneys, and fig. 3 shows an example of this in the medulla. Tubular dilatation without associated fibrosis may have been caused by tubular atrophy. Another possibility is distal obstruction, especially as "calcification", *i.e.* deposition in or around the kidney tubules of cretaceous material characteristically stained by hæmatoxylin and rendered black by the von Kossa silver nitrate stain, and destruction of the papilla tip were noted several times. The papilla-tip lesion is shown in fig. 4 which may be compared with the normal appearance of a papilla tip shown in fig. 5. An identical papillary lesion was reported in the kidneys of essential fatty acid-deficient rats (Borland and Jackson, 1931), and as hæmaturia has often been noted in such rats (*e.g.* by Burr and Burr, 1930, and Birch, 1938) it seemed possible that essential fatty acid-deficiency might have been a factor in the present experiments. However, as none of the inanition or *ad lib.* control rats used in experiments 2 and 3 developed hæmaturia, and as 5 per cent. margarine and 5 per cent. lard had been used respectively in these experiments, it is unlikely that an essential fatty-acid deficiency was actually responsible for the lesions observed in the present experiments. Marked deposition of some calcium-containing compound was often noted in the kidneys of the pyridoxin-deficient hooded rats, and was usually cortico-medullary in distribution (fig. 6). The significance of deposition of calcium compounds in the kidney, however, must be interpreted with care as it may occur in normal control or stock animals. Calcium deposition was frequently observed in many of the control rats in the present experiments but, with the exception of one animal, was never so marked as in fig. 6.

Severe pyelonephritis was observed *post mortem* in two pyridoxin-deficient rats, 1 hooded and 1 albino. Gross erosion, destruction, and

PYRIDOXIN DEFICIENCY

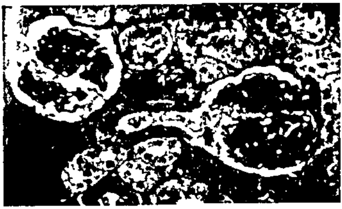

FIG 1 —Kidney of pyridoxin deficient rat, showing deposits in subcapsular spaces and proximal tubule Periodic acid Schiff ×270

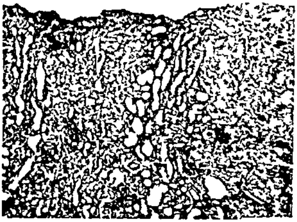

FIG 2 —Kidney of pyridoxin deficient rat, showing areas of fibrosis and tubular dilatation The kidney surface is puckered over the affected areas Hæmatoxylin and eosin ×55

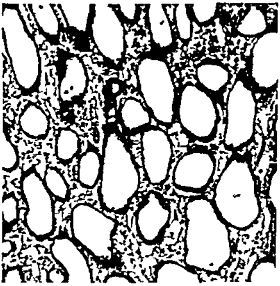

FIG 3 —Kidney of pyridoxin deficient rat, showing tubular dilatation in medulla Hæmatoxylin and eosin ×90

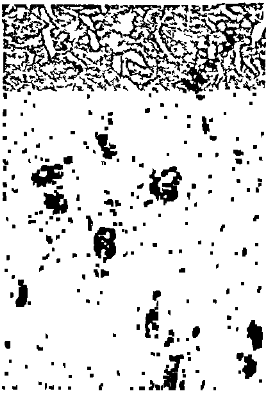

FIG 6 —Kidney of pyridoxin deficient rat showing calcium deposition in tubules of cortico medullary region von Kossa ×50

neutrophil infiltration of the papillæ were present, these lesions were quite different from those described above Fig 7 shows an affected tubule containing a neutrophil cast In two other pyridoxin deficient rats, both hooded, a few neutrophils were seen in an occasional tubule in the renal papilla, and a diagnosis of mild pyelonephritis was made Although several of the pyridoxin deficient hooded rats of the long term experiments 2 4 had severe hæmaturia at the time of death, only an occasional subcapsular space in the kidneys of these animals contained the amorphous material that was the outstanding lesion in the pyridoxin deficient hooded rats of the short term experiment 1

Arteriosclerotic lesions have been described in pyridoxin deficient monkeys by Rinehart and Greenberg (1949) Blood pressures were not recorded in any of the present experiments, but the renal and cardiac hypertrophy observed in most of the pyridoxin deficient rats of both strains (Agnew) suggests that some degree of hypertension may have been present Arteriosclerotic lesions were not, however, seen in any of the blood vessels of the hearts and kidneys examined

## DISCUSSION

Hæmaturia in pyridoxin deficient rats has already been fully discussed (Agnew) Although hæmaturia occurred in all the long-term pyridoxin deficient hooded rats under observation (experiments 2 4, tables I and II), it was stressed in the earlier paper that there may be great variation in the incidence and time of onset of the bleeding It was also observed that although none of the pyridoxin-deficient albino rats developed hæmaturia the hearts and kidneys of these rats, like those of the pyridoxin deficient hooded rats, were usually significantly heavier (g /100 g bodyweight) than those of corresponding inanition and *ad lib* fed controls The degree of severity of pyridoxin depletion, as gauged by the rate of development and severity of acrodynia like skin lesions did not appear to be the factor determining the observed strain differences in susceptibility to the development of hæmaturia

Although hæmaturia was not observed in any of the pyridoxin-deficient albino rats (experiment 5, tables I and II), a few red blood cells were detected microscopically in the urine of the *ad lib* fed and inanition control rats of one trio of albinos The rat normally excretes protein in the urine (Gilson, 1949), and possibly a greater degree of patency of the glomerular membrane than normal might allow the escape of an occasional red cell Occasionally, a few red blood cells may be observed in the tubules of normal rats (fig 8) Microscopic hæmaturia of variable duration has also been reported in apparently normal rats by Cavelti and Cavelti (1945) and by Humphrey (1948), and no significance is therefore attached to the microscopic hæmaturia observed in the albino control rats of experiment 5 Presumably strain differences were responsible for the failure of the pyridoxin-deficient albino rats to develop severe hæmaturia.

Because 4 of the 23 long-term pyridoxin-deficient rats developed pyelonephritis, the possibility that the hæmaturia and renal lesions in the pyridoxin-deficient hooded rats might have resulted from this disease has to be considered. Agnew and Cook confirmed and extended the observation of Stoerk and Eisen (1946) that pyridoxin-deficient rats showed impaired antibody production, but the poor antibody response of these rats to such antigens as sheep erythrocytes or a killed culture of *Bacterium typhosum* does not necessarily mean that the rats would be more susceptible to infection. Occasional spontaneous abscess formation, however, has been observed in pyridoxin-deficient rats, *e.g.* Agnew and Cook, table 3, trio 16. Urine cultures from other pyridoxin-deficient rats with hæmaturia failed to reveal organisms, *e.g. Salmonella enteritidis,* which Smadel (1937) found to be commonly associated with outbreaks of infective pyelonephritis in stock rats. Examination of the kidneys of the pyridoxin-deficient hooded rats of experiment 1 killed shortly after the onset of hæmaturia did not reveal signs of early pyelonephritis and bacteria were not seen in Gram-stained sections. Finally, pyelonephritis was noted in one of the pyridoxin-deficient albino rats but hæmaturia was not observed in any of these, although antibody production was probably impaired. The diagnosis of pyelonephritis rested on the finding of pus cells in the kidney. The gross fibrosis in several of the kidneys might have been, in part, the end result of intercurrent pyelonephritis ; if some of these kidneys had been examined earlier, pus cells might then have been present and hence more instances of pyelonephritis recorded. It seems likely that hæmaturia, induced by deficiency of pyridoxin, caused most of the renal lesions described in the present paper, and that pyelonephritis was responsible only for the presence of pus cells and possibly, in part, for the gross fibrosis.

## SUMMARY

Renal lesions were observed in several pyridoxin-deficient rats and appeared to be the result of hæmaturia. Hæmaturia was not observed in long-term pyridoxin deficiency in albino rats although the kidneys and hearts of these rats, like those of long-term pyridoxin-deficient hooded rats, all of which had hæmaturia, were significantly heavier (g./100 g. bodyweight) than those of corresponding inanition and *ad lib.* fed controls.

The earliest renal lesion detected in hooded short-term pyridoxin-deficient rats with hæmaturia appeared to be one affecting the patency of the glomerular filter. Amorphous, slightly eosinophilic material was deposited in many subcapsular spaces. Protracted hæmaturia resulted in gross calcium deposition, especially in the cortico-medullary zone. Deposition of calcium in and destruction of the renal papilla were observed in several of these rats.

PYRIDOXIN DEFICIENCY

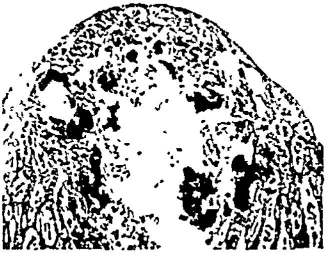

FIG. 4.—Kidney of pyridoxin-deficient rat, showing calcium deposition in and destruction of papilla tip. The desquamated epithelial cells present in most of the tubules are the result of post-mortem change. (Compare normal appearance in fig 5). Hæmatoxylin and eosin. ×45.

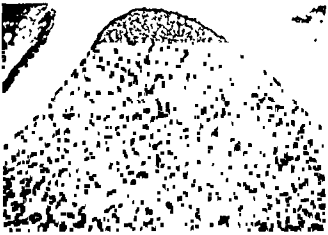

FIG. 5.—Longitudinal hemisection of renal papilla of normal rat. Hæmatoxylin and eosin ×55

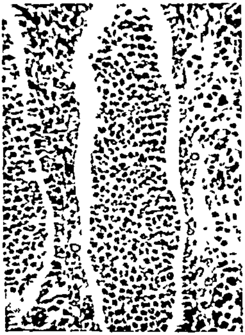

FIG. 7.—Neutrophil cast in tubule of kidney of a pyridoxin deficient rat Hæmatoxylin and eosin. ×220.

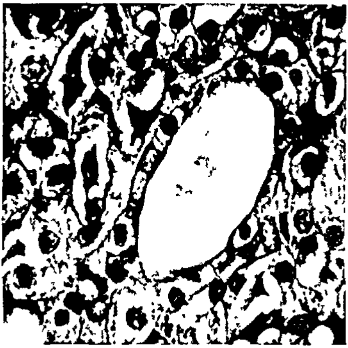

FIG. 8.—Kidney of normal control rat showing 5 red blood cells in a tubule. Hæmatoxylin and eosin ×620

Several grossly scarred kidneys were observed in pyridoxin deficient rats but the marked fibrosis in these kidneys might have resulted, in part, from intercurrent pyelonephritis Four obvious examples of pyelonephritis were observed in pyridoxin deficient rats but this disease is not thought to have caused the hæmaturia observed in 22 of 24 pyridoxin deficient hooded rats The significance of pyelonephritis is discussed with reference to antibody production in pyridoxin deficient rats

Part of this work was done during the tenure of a full time Agricultural Research Council scholarship I wish to thank Mr G Porter and his staff for their skilful and careful handling of the animals, Mr E A S Rattray for expert technical assistance, and Mr F Morrico for the photographs

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576 . 809 . 729 : 576 . 851 . 31 (*Vibrio choleræ*)

# AN EXPERIMENTAL STUDY OF THE ACTION OF CHOLERA TOXIN

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(Plates CXXIII and CXXIV)

The exact pathogenesis of cholera, the classical scourge of Bengal, is debatable even to this day. Apparently the causative organisms remain confined to the lumen of the intestinal tract, with occasional access through its epithelium, but not beyond the basement membrane (Wilson and Miles, 1946) and there is a vague belief that the cholera evacuations are the result of irritation of the intestinal mucosa by the vibrios. More recently Burrows *et al.* (1944) have endeavoured to show that cholera endotoxin greatly increases the permeability of isolated strips of intestine from normal guinea-pigs and rabbits.

Very little attention has been paid to the systemic pathology of the disease, for the extreme dehydration and acid-base imbalance resulting from vomiting and dehydration distract the attention of the observer. Thus, the oliguria and anuria as well as the kidney changes have been attributed to these factors. However, the toxic appearance of the cholera patient, the occurrence of cases of *cholera sicca* with profound collapse but few evacuations, and the persistence of anuria with death therefrom in many cases that have reached the reaction stage, very naturally suggest that the disease in man is primarily an intoxication and that the organisms multiply in the lumen of the gut, die, undergo autolysis and liberate their endotoxin which acts locally and is also absorbed, producing systemic effects.

To test this conception, we have studied the pathological effects of intraperitoneal injection of a suspension of heat-killed cholera organisms into rabbits. Preliminary observations on some of the renal changes have been communicated to the first annual conference of the Indian Association of Pathologists.

## Methods

(a) *Preparation of the toxic material.* Freshly voided fæces of a typical patient seized with cholera at the commencement of an epidemic was seeded directly on a McConkey's plate which yielded a pure culture on twenty-four hours' incubation. The organism was tested for its reaction to Gram staining, motility, sugar reactions, hæmolysis and agglutination and was identified as an Inaba strain of *Vibrio choleræ*. From the plate several nutrient broth tubes were inoculated ; the latter were then used to inoculate an agar medium prepared in Roux flasks. Each of these second subcultures, after incubation for 24 hours, was washed with sterile normal saline, pooled and centrifuged to throw down the bacteria. The bacterial deposit was suspended in an equal volume of sterile normal saline and the number of bacteria per c.mm. was counted with the help of a hæmocytometer, using warm dilute carbol fuchsin as the diluent, and was found to be 100 million organisms per c.mm. The suspension was then sterilised by heating it in a water bath at 56° C. for 15 minutes and two control broth tubes were inoculated from it to confirm its sterility. This short exposure is sufficient to kill *Vibrio choleræ* (Kitasato, 1888-89) and might be expected to interfere little with the toxicity of the preparation. The suspension was stored in the refrigerator and used as the toxic material whenever required. Before use, however, it was warmed in the incubator for 15 minutes and then given a thorough shaking. It is obvious that it contained the bacterial bodies and their endotoxin although we refer to the preparation as " cholera toxin ". An emulsion of heat-killed typhoid bacilli has been designated " typhoid toxin " in a similar sense.

For ascertaining the toxicity of the suspension, healthy male rabbits, 1 to 1·5 kg. body weight, were employed. As rabbits are known to store extra water in the tissues (Cameron and Courtice, 1946-47) they were given no water for two days and no food for one day before the experiments. By trial it was found that an intraperitoneal dose of 1 c.c. per kg. of the suspension regularly killed the animal in about 2 hours. This appeared to be the minimum lethal dose. Toxicity was maintained in the refrigerated suspension for 4 months.

(b) *Experimental procedures.* Seven animals received a minimum lethal dose of the toxic preparation (1 c.c. per kg.). Hæmoglobin percentage, R.B.C. count and hæmatocrit value of blood obtained from the ear veins were estimated half-an-hour before and every half-hour after the injection of the toxin. In moribund animals, blood samples were collected before the completion of the last half hour or terminally by cardiac puncture. About 0·5 c.c. of blood was run into test tubes containing double oxalate as anticoagulant and was mixed gently. Hæmoglobin percentage was determined with a Hellige neoplan hæmoglobinometer, red blood cells were counted with a Neubauer hæmocytometer and packed cell volume was measured by spinning in a pair of Wintrobe's capillary hæmatocrit tubes for half-an-hour at 3000 *r.p.m.* Blood pressure was recorded from the central artery of the ear by means of a Grant capsule (Grant and Rothschild, 1934) just before the blood samples were collected.

A post-mortem examination was made immediately after death and fluid from the peritoneal cavity was collected, measured and centrifuged. A smear from the deposit was stained with Leishman's stain and the nature of the cells was ascertained. Prof. H. Saha kindly examined the supernatant fluid for its total protein content by the photoelectric method of Greenberg. Tissues from the heart, lungs, liver, kidneys, suprarenal, stomach, small intestine, large intestine and appendix were fixed immediately in 10 per cent. formol saline, embedded in paraffin and sections were stained with Ehrlich's acid hæmatoxylin and eosin.

One kidney from each animal was removed, together with its fatty capsule, after the pedicle had been tied to avoid loss of blood. After fixing in formol saline for 24 hours it was bisected along the convex border. It was further fixed for about a week, when 100 μ thick frozen sections were made parallel to the cut surface and stained by Lepehne-Pickworth's method for showing up the blood vessels. This method has been often used by one of us (De, 1950) and by other workers (Campbell *et al.*, 1938 ; Blackwood, 1941)

Cholera toxin

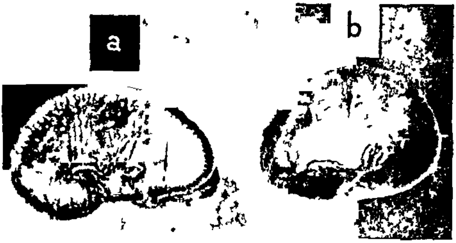

FIG 1—Macroscopic appearance of cut surface of rabbit kidney, (a) from animal which received 1 m l d of cholera toxin, showing pallor of the cortex and congestion of the medulla and (b) from control animal showing uniform appearance of cortex and medulla

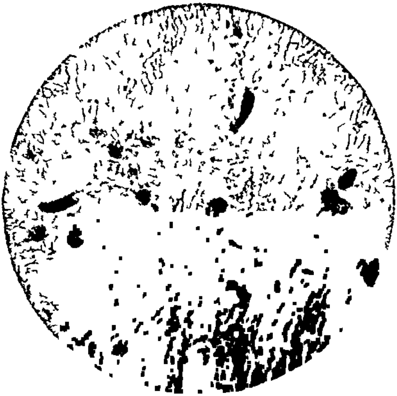

FIG 2—Vascular pattern of parts of cortex, medulla and boundary zone of kidney from a rabbit which received 1 m l d of cholera toxin intraperitoneally The cortical glomeruli are ischæmic and ghost like in appearance The juxta medullary glomeruli are distinct and parallel Closely set vessels (vasa recta) are prominent in the medulla Frozen section—100 μ thick Pickworth s stain × 15

CHOLERA TOXIN

FIG. 3.—Vascular pattern of parts of cortex, medulla and boundary zone of kidney from a control animal Both the cortical and juxta-medullary glomeruli are distinct. *Vasa recta in the medulla do not appear to be so prominent or so closely set.* Frozen section—100 $\mu$ thick. Pickworth's stain. × 15.

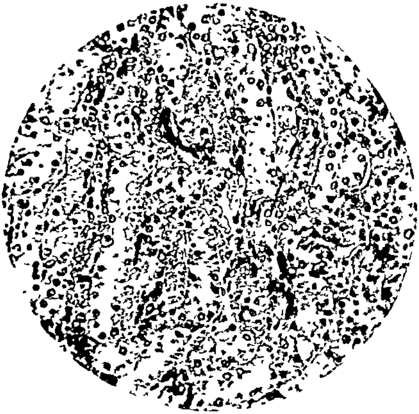

FIG. 4.—Section showing extreme cloudy swelling and necrosis of tubular epithelium in the renal medulla. Hæmatoxylin and eosin. × 250.

for the demonstration of pathological changes in the vascular pattern of the brain

The above techniques were employed with animals which received —

(1) sublethal dose of cholera toxin (0 75 c c per kg ) intraperitoneally—5 animals
(2) high fatal dose of cholera toxin (5 c c per kg ) intraperitoneally—5 animals
(3) sublethal dose of typhoid toxin (1 c c per kg of 300 millions of organisms per c mm )—5 animals
(4) high fatal dose of typhoid toxin (3 c c per kg of 300 millions of organisms per c mm )—5 animals
(5) sterile hypertonic glucose (10 c c per kg of 60 per cent solution) *subcutaneously in the thigh—5 animals*
(6) sterile normal saline (1 c c per kg ) intraperitoneally—3 animals

Those animals which survived more than two hours were killed with chloroform 3 hours after the injection and their organs were studied macroscopically and microscopically

In order to trace the path of distribution of fluid leaving the blood vessels, 4 c c of a 2 per cent solution of Evans blue in normal saline sterilised by steam, was injected into the ear vein of two pairs of rabbits (Seneviratne, 1948) One of the pairs had received 1 c c per kg of the cholera toxin into the peritoneum one hour beforehand, while the other had received the same dose of normal saline and served as a control The dye behaves like serum albumin in so far as the permeability of membranes is concerned (Rawson, 1942 43) The animals were killed with chloroform 45 minutes later and the distribution and the density of the blue colour were studied in all instances

## RESULTS

### (1) *Experiments with cholera toxin*

The blood pressure changes and the hæmatological findings of the 7 rabbits which died within 2 hours of injection of 1 c c per kg of the emulsion of cholera vibrios are summarised in the table There was progressive fall of blood pressure, which commenced within half an hour after the injection and continued till death The hæmoglobin percentage did not rise till one hour after the injections when the red blood cell count and packed cell volume also showed a corresponding increase

About 5 6 c c of peritoneal fluid was obtained *post mortem* from each animal It was clear or slightly cloudy and in one case was frankly sanguineous In two animals there was evidence of clotting in the form of a thin film within *the fluid The centrifuged deposit disclosed in all animals a preponderance* of red blood cells (minimum 90 per cent ) a few mononuclear cells (7 per cent ) and fewer polymorphs (maximum 3 per cent ) The protein content of the supernatant fluid from two animals which showed colourless peritoneal exudate was 3 8 per cent

Besides fluid in the peritoneum and some congestion of the abdominal organs the small intestine was found to be soft and œdematous and it contained a little pale jelly like fluid, while the large intestine often contained hard scybalous fæcal matter When

TABLE—*Summary of results*

| Time interval | B.P. (mm. Hg.) | Hæmoglobin (per cent. Hellige) | Red cell count (millions per c.mm.) | Packed cell volume (per cent.) |
|---|---|---|---|---|
| **Effect of intraperitoneal injection of a lethal dose (1 c.c. per kg. B.W.) cholera toxin (means of 7 animals)** | | | | |
| ½ hour before injection | 69 | 107 | 7·2 | 37 |
| ½ hour after injection | 60 | 106 | ... | ... |
| 1 ,, ,, ,, | 42 | 112 | 8·4 | 39 |
| 1½ hours ,, ,, | 30 | 110 | ... | ... |
| 2 ,, ,, ,, | 15 | 113 | 8·1 | 40 |
| **Effect of intraperitoneal injection of a sublethal dose (0·75 c.c. per kg. B.W.) cholera toxin (means of 5 animals)** | | | | |
| ½ hour before injection | 60 | 110 | ... | ... |
| ½ ,, after injection | 40 | 114 | ... | ... |
| 1 ,, ,, ,, | 30 | 106 | ... | ... |
| 1½ hours ,, ,, | 55 | 108 | ... | ... |
| **Effect of intraperitoneal injection of heavy lethal dose (5 c.c. per kg. B.W.) cholera toxin (means of 5 animals)** | | | | |
| ½ hour before injection | 60 | 116 | ... | ... |
| ½ ,, after injection | 20 | 118 | ... | ... |
| 1 ,, ,, ,, | 17 | 125 | ... | ... |
| 1½ hours ,, ,, | dead | | ... | ... |
| **Effect of intraperitoneal injection of 1 c.c. per kg. B.W. normal saline (means of 3 animals)** | | | | |
| ½ hour before injection | 65 | 97 | 5·8 | 34 |
| ½ ,, after injection | 68 | 95 | ... | ... |
| 1 ,, ,, ,, | 65 | 97 | 6·2 | 34 |
| 1½ hours ,, ,, | 63 | 95 | ... | ... |
| 2 ,, ,, ,, | 60 | 93 | 5·9 | 33 |
| 3 ,, ,, ,, | 60 | 93 | ... | ... |
| **Effect of subcutaneous injection of 30 c.c. per kg. 60 per cent. glucose (means of 5 animals)** | | | | |
| ½ hour before injection | 71 | 95 | 6·1 | 33 |
| ½ ,, after injection | 70 | ... | ... | ... |
| 1 ,, ,, ,, | 51 | 103 | 6·73 | 36 |
| 1½ hours ,, ,, | 60 | 110 | ... | ... |
| 2 ,, ,, ,, | 63 | 116 | 7·5 | 41 |
| 3 ,, ,, ,, | 51 | 122 | 9·6 | 43 |
| **Effect of intraperitoneal injection of a heavy lethal dose (3 c.c. per kg. B.W.) typhoid toxin (means of 5 animals)** | | | | |
| ½ hour before injection | 63 | 105 | 6·5 | 35 |
| ½ ,, after injection | 58 | 100 | 6·7 | 34 |
| 1 ,, ,, ,, | 20 | 106 | 6·3 | 34 |
| 1½ hours ,, ,, | 16 | 108 | 6·3 | 35 |
| 2 ,, ,, ,, | ... | 106 | 6·8 | 34 |
| **Effect of intraperitoneal injection of sublethal dose (1 c.c. per kg. B.W.) typhoid toxin (means of 5 animals)** | | | | |
| ½ hour before injection | 62 | 100 | ... | ... |
| ½ ,, after injection | 40 | 102 | ... | ... |
| 1 ,, ,, ,, | 40 | 104 | ... | ... |
| 1½ hours ,, ,, | 20 | 98 | ... | ... |
| 2 ,, ,, ,, | 52 | 102 | ... | ... |

the kidneys were split along their convex border, their cut surfaces in six rabbits disclosed an extremely pale cortex while the medulla and the boundary zone appeared a dark, dusky red colour (fig. 1a). We were reminded of the coloured diagram of cholera kidney depicted by De and Chatterjee (1935), of the appearance of the kidney of traumatic anuria illustrated by Bywaters and Dible (1942) and of the kidney appearance obtained after stimulation of nerves by Trueta *et al.* (1946).

Definite microscopic changes were found in the following organs.

*Kidney.* Pickworth's stain showed ischæmia of the cortical glomeruli and poor vascularity around the cortical tubules. These changes were most marked and most constant in the more superficial parts and their extent deeper down was variable. In the extreme form, only the juxta-medullary glomeruli seemed to escape this ischæmic change (figs. 2 and 3) and from their efferents numerous pencils of prominent vessels, the so-called vasa recta spuria, could be seen passing down the medulla.

Most of the cortical glomeruli appeared collapsed, only an occasional red blood corpuscle showing in the tufts. The spaces of the Bowman's capsules and the tubular lumina were almost non-existent. The peri-glomerular convoluted tubules appeared perfectly healthy as also the medullary rays. A few venous channels could be detected but they contained only clear plasma.

The glomeruli in the boundary zone, especially those in the two innermost rows were, in contrast to the above, obviously congested. Their capillary tufts were open and filled with blood cells. The peri-glomerular convoluted tubules, both proximal and distal, showed granular and more eosinophilic cytoplasm, the inner margin of the cells tending to be irregular but the nuclei healthy. The Bowman's capsules and the tubules in this region were patent.

At the medullary region, intense congestion of the vasa recta coursing between the straight tubules was striking. The cells of the limbs and loops of Henle were swollen and granular while parts of the cells nearest to the lumen had disappeared. According to Karsner (1935) these changes represent advanced cloudy swelling of the renal epithelium (fig. 4). Nuclei were pyknotic and karyolytic at places. The cells of the collecting tubules were normal.

*Heart.* The muscle fibres were well separated from each other. This was very striking when compared with the heart muscle obtained from rabbits injected with hypertonic glucose solution or with emulsion of killed typhoid bacilli or from the control animals. The cardiac muscle fibres exhibited swelling and granular degeneration of the cytoplasm and at places loss of striation was evident. There was no sign of necrosis or hæmorrhage in the cardiac walls.

*Small intestine.* The submucous and serous coats showed clear evidence of œdema in the form of wider spaces between the connective tissue fibres. The stroma of the mucous membrane also appeared œdematous but the epithelial lining was normal. No cellular exudate was present in any of the coats.

With small doses (0·75 c.c. per kg.) of the cholera toxin, changes in vascular pattern of the kidneys were not so prominent, although similar parenchymatous and glomerular changes could be detected. With heavy lethal doses (5 c.c. per kg.), the cortex was very congested and the cortical peri-glomerular tubules showed parenchymatous damage similar to that present in the medullary and juxta-medullary tubules. With a sublethal dose, there was a temporary fall of blood pressure which returned towards normal in 1½ hours. Hæmoconcentration was not marked. With a heavy lethal dose, there was marked fall of blood pressure till the animal was dead in 1½ hours' time and there was considerable hæmoconcentration after 1 hour (table).

In the animals which received cholera toxin intraperitoneally followed by Evans blue intravenously, the peritoneal fluid was coloured intensely blue. The serous coats of the small and large intestines and of the appendix were a deeper blue than in the control animals. The contents of the small intestines (both jejunum and ileum) were fluid and appeared distinctly blue but were uncoloured in the control animals. The heart muscle in both groups stained deep blue but careful examination suggested a deeper colour in the test animals.

### (2) *Experiments (control) with normal saline*

The table shows that the mean blood pressure, red cell count, hæmoglobin and packed cell volume remained almost constant throughout the period of experiment in 3 control rabbits. Little fluid was found in the peritoneal or other cavities and no abnormality could be detected in any of the organs.

### (3) *Dehydration experiments with hypertonic glucose solution*

The table summarises the mean changes in blood pressure and in the blood of 5 rabbits which received hypertonic glucose solution subcutaneously, the method used by Davis (1940, 1941), Stoner and Green (1945), Cameron *et al.* (1946) and Cooray and De (1949) for the study of some effects of simple dehydration. We have confined our observations to the first three hours of the experiments because we wished to find out if simple dehydration was responsible for the results of our short-lasting (*ca.* 2 hours) experiments with cholera toxin. There was considerable hæmoconcentration within this time, as indicated by the rise in hæmoglobin percentage, red cell count and packed cell volume, much more than with cholera toxin, but the blood pressure showed a much smaller and less regular fall.

The organs of these animals appeared congested when killed while the cut surface of the kidney presented a uniform congested appearance; the medulla was not so densely congested, nor was the cortex so pale as in the animals injected with cholera toxin. No microscopic abnormality was detected in any of the organs except congestion.

### (4) *Experiments with typhoid toxin*

The effects of intraperitoneal injections of a rather heavy lethal dose and of a sublethal dose of an emulsion of killed typhoid bacilli on blood pressure, hæmoglobin percentage, red blood cell count and packed cell volume of peripheral blood are shown in the table. It will be noted that in both cases the blood pressure continued to fall, not unlike the behaviour observed with cholera toxin. Usually after 2 hours either the blood pressure was so low that it could not be measured and the animal died, or the blood pressure rose towards normal, as with the sublethal dose of cholera toxin; on the other hand, no hæmoconcentration as judged by rise in hæmoglobin level was observed.

At autopsy, free fluid was not found in the peritoneal cavity nor were there any other macroscopic abnormalities. The cut surface of the kidneys of the animals presented a uniformly congested appearance of both the cortex and the medulla.

The peri-glomerular convoluted tubules, both in the superficial cortex and in the boundary zone, and the limbs and loops of Henle in the medulla exhibited cloudy swelling of the epithelium, more markedly and more uniformly with the fatal dose. Histological examination revealed a complete absence of œdema in the myocardium and intestinal coats although the heart muscle showed some granular degeneration and loss of striation.

## DISCUSSION

Toxic effects resulting from the intraperitoneal injection of washed saline suspension of killed vibrios must be due to contained endotoxin. The local peritoneal reaction to this toxin is engorgement of vessels and an outpouring of fluid which is poor in cells and rich in protein. The fluid does not strictly represent a true inflammatory exudate as the leucocytes, especially the polymorphonuclear cells, are so scanty. On the other hand, (i) preponderance of red blood cells, (ii) high protein content approaching that of plasma with the presence of fibrinogen and formation of membranous clot and (iii) frank leakage of Evans blue into this peritoneal fluid suggest a grave alteration in the permeability of the capillaries. The toxin has so injured their walls that they allow almost free passage of plasma as well as of some red blood cells. Such a toxic injury to capillary walls is not unknown with bacterial products (Seneviratne, loc. cit.) though it may be noted that introduction of a sterile emulsion of typhoid bacilli has not given such an outpouring of fluid. This suggests that the local alteration of capillary permeability as observed with cholera toxin is not a non-specific reaction of the peritoneum to bacterial products. The loss of fluid from the circulation is obviously responsible for the hæmoconcentration. Histological and dye studies show that this fluid loss is not confined locally to the peritoneum but affects other tissues. With the emulsion of typhoid organisms, lack of hæmoconcentration seems quite compatible with absence of extravasation of plasma into the peritoneum.

The diminution in volume of circulating blood which is suggested by the hæmoconcentration might well be blamed for the fall of blood pressure observed with cholera toxin. But, on closer scrutiny and especially on comparison with the results of subcutaneous injection of hypertonic glucose solution, it becomes obvious that a much higher degree of hæmoconcentration is possible without any considerable effect on blood pressure. This leads us to think that hæmoconcentration produced by cholera toxin may have contributed to the observed fall of blood pressure but is not chiefly responsible for it. There may have been some more direct effect of the toxin on the cardiovascular system because of its absorption into the general circulation. Ample support for this view exists in the form of histological changes in the heart, kidneys and intestines. We would suggest that the œdema fluid separating individual muscle fibres of the heart mechanically interferes with their contraction and thereby impairs the functional efficiency of the myocardium. The parenchymatous degeneration of the muscle too no doubt affects its function. These structural changes in the myocardium would contribute to the observed fall of blood pressure. Further evidence comes from the work of Manwaring *et al.* (1923) who observed extensive heart œdema when they perfused the isolated organ with saline mixed with filtrate of old broth culture of *Vibrio choleræ*. They recorded functional impairment of the muscle contractions which they attributed to cardiac œdema.

We also think that the submucous œdema of the small intestine must have been due to specific action of the cholera toxin after its absorption into the general circulation. The toxin specifically damaged the capillaries of this area, increasing their permeability to protein. This is suggested by the deep blue colour of the wall of the small intestine and also by a bluish colouring of its contents observed after the intravenous injection of Evans blue.

The renal changes deserve comment. The cortical pallor and the medullary congestion resulting from sublethal and minimum lethal doses of cholera toxin are reminiscent of the changes produced by nervous stimulation (Trueta *et al.*, 1947) and may indicate a similar mechanism of spasm of interlobular arteries and afferent arterioles and diversion of the cortical blood through juxta-medullary glomeruli to the medulla.

The kidney also exhibited evidence of toxic damage to the parenchyma in the form of severe cloudy swelling and necrosis of the tubular epithelium in the boundary and medullary zones where the toxin-laden blood had been diverted The cortex was thus deprived of blood and so saved from the toxin The peri-glomerular convoluted tubules in this region appeared healthy and had not suffered from the effects of ischæmia The reason for this, no doubt, is the short duration of the ischæmia which lasted for about 2 hours, whereupon the animal died Franklin (1950) remarks that the cortical elements of the rabbit can stand an ischæmia of about 4 hours, and this fact may well explain the absence of anoxic damage here

With heavy doses of cholera toxin, however, the mechanism appears to fail, and the toxic blood finds a passage to the cortex, thereby *injuring the convoluted tubules With typhoid toxin, given either* in lethal or in non-lethal doses, this mechanism does not come into play to protect the cortex

Our experimental findings explain reasonably well the chain of events that takes place in human cases of cholera The endotoxin, liberated by the death and disintegration of the vibrios, through a local action on the capillaries of the intestinal mucosa, increases their permeability causing an outpouring into the lumen of the gut of plasma sometimes mixed with red blood cells The toxin is also absorbed and thereafter exerts a specific toxic effect on the submucosal and mucosal capillaries of the small intestine The loss of fluid from the circulation results in hæmoconcentration and this undoubtedly contributes to the fall of blood pressure especially when this loss is continued for some considerable time But the more important cause of the shock-like manifestation lies in the effects of the toxin on the cardiovascular system One of these toxic effects is directed against the myocardium, where the specific action on the myocardial capillaries gives rise to œdema and impairment of function The occurrence of cases of *cholera sicca* with pronounced shock but little purging strongly suggests the predominant role of the absorbed toxin on the cardiovascular system The toxin also acts specifically on the renal vessels and parenchyma, leading to the same kind of shunting mechanism that we have demonstrated in the rabbit It may well be that in the cholera cases which recover from the stage of shock and oliguria the protective mechanism has been successful In those that lapse later into the state of postcholeraic uræmia, toxic and ischæmic damage of the cortical parenchyma is irreversible, due either to failure of this mechanism because of heavy amounts of toxin or to long duration of the ischæmia

## SUMMARY AND CONCLUSIONS

Intraperitoneal introduction of suitable amounts of cholera toxin in the form of heat killed washed suspension of *Vibrio choleræ* kills rabbits in about 2 hours The injection is followed by hæmoconcentration and by fall of blood pressure which is due partly to the degree of hæmoconcentration and partly to a direct action on the cardiovascular system of the toxin after it is absorbed

Hæmoconcentration is the result of outpouring into the peritoneum of fluid which is rich in protein and poor in cells. Microscopic evidence of œdema is also seen in the myocardium and in the mucosa and submucosa of the small intestine. Evans blue given intravenously leaks into all of these places. These facts suggest an alteration of permeability of the capillaries which allows passage of plasma-like fluid into the tissues. Such a change appears to be brought about by direct local action of the cholera toxin and also by its remote specific action on the capillaries of the myocardium and of the inner coats of the small intestines.

The toxin also affects the vasculature and the parenchyma of the kidneys, leading to diversion of the toxin-laden blood from the cortex to the medulla. The vital cortical elements are thus protected from the effects of the toxin which, however, damages those parts of the nephrons situated in the medulla and juxta-medullary regions. As the majority of the glomeruli are rendered ischæmic, filtration of urine is diminished and further loss of body fluid by this route is prevented. This protective mechanism fails when a very heavy dose of cholera toxin is injected. The significance of these experimental results in relation to the human disease is discussed.

We wish to thank Professor H. Saha for estimating the protein content of peritoneal fluid, Dr K. P. Sen Gupta for some technical help, Drs A. R. Roy and H. K. Chakravarty for some helpful criticism and Mr J. Saha for the photomicrographs.

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# TWO CASES OF RENAL CARCINOMA SHOWING CYTOPLASMIC INCLUSIONS

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(Plate CXXV)

Carcinoma of the renal parenchyma is a fairly frequent disease in the human subject, comprising about 2·5 per cent. of all malignant tumours occurring between the ages of thirty and seventy. Two examples have recently been encountered in widely separated parts of the country, in which the malignant cells contained cytoplasmic inclusions bearing a certain resemblance to inclusion bodies of virus origin. Since no report of a similar finding in this common disease has been found in the literature, it appears worth while to place on record a detailed account of these neoplasms.

## Case Reports

### Case I

### *Clinical history*

Mrs J. L., aged 41, was admitted to the Glasgow Western Infirmary suffering from mitral stenosis. Fourteen days after admission she complained of constant gnawing pain in the left loin. A firm irregular mass was palpable in the left hypochondrium, mobile and tender, and moving with respiration The urine showed numerous red cells, but no casts or excess of leucocytes. A provisional diagnosis of renal tumour was made Retrograde pyelography showed a filling defect of the left renal pelvis and at the pelvi ureteral junction, suggestive of a neoplasm.

At operation a tumour was found to occupy most of the lower half of the kidney, which was adherent to the peritoneum in this situation. The renal pelvis was slightly distended by a soft mass of growth and, in its upper third, the ureter was adherent to the psoas muscle in which a nodule about the size of an almond was felt. The kidney was removed with 75 mm. of the ureter, and the nodule in the psoas was excised. At no time before or after operation was there any evidence of urinary infection, and the patient made an uneventful recovery.

### *Description of specimen*

The kidney weighed 260 g. In the lower pole was a firm nodular mass, occupying the entire thickness of the organ. On dissection, the tumour was seen to be roughly spherical and 55 mm. in diameter.

It was sharply demarcated by a capsule of compressed and fibrosed renal tissue. The cut surface showed golden-yellow areas of central necrosis. There was no cyst formation or mucoid change. A few fibrous trabeculæ traversed the growth. The tumour extended into the renal pelvis, and there were white papillomatous outgrowths from its lining epithelium. The lumen of the ureter was patent, and no extension along the renal vein was seen.

### *General histological features*

Blocks were taken from the central and peripheral parts of the tumour, from the adjacent tumour-free renal tissue, from the pelvis and ureter and from the nodule in the psoas muscle. The tissues were fixed in corrosive formol and 10 per cent. formol saline. Paraffin and frozen sections were prepared.

Both the architecture of the growth and the degree of epithelial differentiation varied in different parts. The predominant structure was papilliform, and this was most highly developed in the peripheral parts, where the tumour was divided into lobules by fibrous septa derived from the capsule of compressed and fibrosed renal tissue. Elsewhere, the cells were arranged in tubular form. Extensive areas of necrosis with heavy polymorph infiltration were present in the central parts of the growth. Immediately adjacent to the necrotic areas there was an increase in the collagen of the stroma and infiltration with polymorphs. Thin-walled blood vessels were present at the periphery, but the main bulk of the tumour showed low vascularity.

The degree of differentiation in both the papilliform and tubular areas may conveniently be divided into three grades :—(1) areas with well defined epithelium, in which the cells show clearly defined cell membranes and are generally in single layers, but with fairly frequent reduplications. The intervening stroma is very delicate, and of low vascularity, so that the epithelial layers of each papilliform process are separated by little more than the basement membrane. (2) Areas with poorly differentiated epithelium showing great irregularity and heaped into multiple layers in which cell membranes are not distinguishable, but in which the basic papilliform or tubular structure is retained. The stroma in these areas is more abundant, and frequently contains bulky aggregations of very large foamy cells with small dense nuclei. (3) Frankly anaplastic areas in which the features of papilliform or tubular structure are lost, and which show marked nuclear aberrations and mitotic figures. The latter features are readily found only in such areas of maximum anaplasia.

Sections of the pelvi-ureteral junction and of the ureter showed lymphatic permeation of all the layers by malignant cells accompanied by considerable polymorph infiltration.

The malignant cells in the nodule from the psoas muscle showed no suggestion of papilliform or tubular structure, and had a much more abundant fibrous stroma, freely infiltrated with polymorphs

### *The cytoplasmic inclusions*

In the *primary tumour, the cytoplasm of the epithelial cells* unaffected by necrosis contained highly refractile eosinophilic bodies Almost every cell had one or more of these inclusions, producing a very striking microscopic picture The individual bodies varied considerably in shape and size, ranging from 2 15 $\mu$ in their greatest diameter, with an average size rather less than that of the cell nuclei. Only the smallest of the inclusions were spherical All the larger bodies showed various degrees of distortion—elongation, compression and, whenever closely applied to the nucleus, indentation These appearances were taken to indicate a relatively low density and low fluid tension Almost without exception, the cells containing cytoplasmic inclusions had a large eosinophilic nucleolus

The distribution of the bodies varied in different parts of the growth In the areas with well differentiated epithelium, single cytoplasmic bodies of uniform size were localised at the base of the cell between the nucleus and the basement membrane This arrangement was best seen in the papilliform areas with a single layer of epithelium (fig. 1) The size of the inclusions in these areas was generally a little less than that of the nuclei In the areas with poorly differentiated epithelium in multiple layers, there was a corresponding multiplicity of inclusions There was also a greater variation in size, and a greater number of smaller forms, frequently with several bodies in a single cell (fig 2) In the most anaplastic areas with large cells and large aberrant nucleoli, the inclusions were proportionately larger and more distorted Numbers of smaller forms were again often present in association with the larger bodies within a single cell Where the tubules were small and closely packed, with low cubical type of epithelium, the basal location of the bodies was much less in evidence, and they were found more often lateral to the nucleus, and frequently occupied the whole depth of the cell

No organised internal structure was detected in the cytoplasmic bodies With some staining methods the bodies were not homogeneous, but they never suggested the regular structural pattern of a group of elementary bodies

On approaching the necrotic areas, a sharp transition was observed from cells containing inclusions and prominent nucleoli to cells showing neither of these features In some instances nucleoli persisted in the zone beyond where the inclusions disappeared, but in general the absence of both was parallel to a striking degree The majority of these cells were still apparently viable and formed a zone of varying width surrounding the necrotic areas In these zones, the collagen of the stroma was more abundant. Occasionally, the transition was so abrupt as to be a clearly defined line, but this may represent merely the point of contact of two lobules of tumour tissue having separate vascular supplies, one of which was failing. In the same way, there were occasional narrow prolongations of inclusion-bearing cells between two areas of necrosis.

In general, the greatest concentration of inclusions was found at the periphery of the tumour. At the extreme periphery, occasional areas were found where the entire cytoplasm of the cells was occupied by eosinophilic refractile material. In a very few places similar material was found extracellularly. The inclusions were wholly confined to the tumour and none was found in the renal substance proper.

Neither inclusions nor nucleoli were present in the tumour cells in the renal pelvis and ureter or in the nodule from the psoas muscle. In these sites the malignant cells were morphologically similar to those lacking nucleoli and inclusions in the primary tumour, namely, the cells adjacent to necrotic foci. The secondaries also showed marked polymorph infiltration.

## Case 2

### *Clinical history*

Mr A. J. M., aged 46 years, was referred to Ashford Hospital in December 1949, with a history of severe cough of five months' duration, accompanied by yellow non-offensive sputum, shortness of breath and fever, but no hæmoptysis. He had been investigated as a possible case of pulmonary tuberculosis but X-ray examination of his chest suggested silicosis, though there had been no occupational risk. His sputum was consistently devoid of tubercle bacilli and the Mantoux reaction was negative in all dilutions.

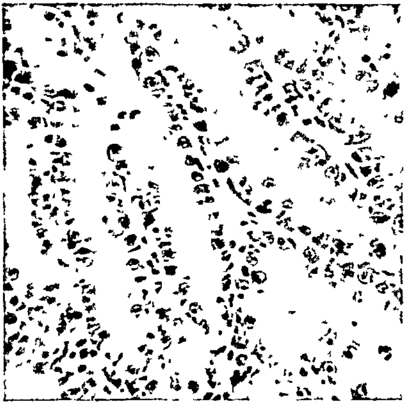
In view of the condition of the patient, treatment was merely symptomatic, and he was discharged to attend the outpatient department. He was admitted to an associated hospital three weeks after his discharge, with a diagnosis of bronchopneumonia and died three weeks later.

### *Post-mortem findings*

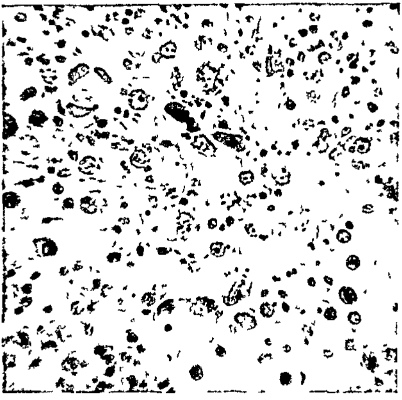
The *right kidney* contained a large tumour, 8×8×6 cm. in the upper pole, unattached to surrounding structures. On cutting, it showed much central fibrosis and areas of cyst formation. Its colour was yellowish-white, with some brownish areas of necrosis and hæmorrhage. The tumour was covered by the renal capsule, and was demarcated from the kidney by compressed renal substance. There was no involvement of the renal pelvis or vein. All the abdominal lymph nodes were neoplastic and continuous with those in the thorax. The rest of the urogenital tract was normal.

There was a little free fluid in the peritoneal cavity. Apart from an old healed duodenal ulcer, the alimentary tract was normal. The spleen was "toxic". The pancreas and adrenals were normal. The liver contained a small metastasis in the right lobe and one in the left. The gall-bladder and bile-ducts were healthy. The left kidney was congested but otherwise normal.

CYTOPLASMIC INCLUSIONS IN TUMOUR CELLS



CASE 1.—Well differentiated papilliform growth with predominantly subnuclear localisation of the cytoplasmic inclusions Phloxin tartrazine ×350



CASE 2—Pleomorphic tumour growth with corresponding variation in the cytoplasmic inclusions Phloxin tartrazine ×350

There were some small neoplastic lymph nodes in the deep cervical chain, otherwise no abnormalities in the neck. *Thorax.* Bilateral pleural effusion of about one litre was present with fibrinous adhesions over the lower lobe of the left lung. The parietal pleura showed scattered firm nodules from 5-10 mm. diameter, very numerous over the diaphragm ; some were pedunculated. The lungs were almost completely solid, showing generalised multiple white discrete tumour nodules and intervening bronchopneumonia. The nodules were mostly rounded or lobulated and their size varied from 2-15 mm. diameter. Peribronchial spread was prominent. All the mediastinal lymph nodes were neoplastic, but were discrete. Nodules were scattered over the outer surface of the pericardial sac. The epicardium and the heart showed no abnormality.

### *General histological features*

A carcinoma which for the most part was growing in masses with no attempt at differentiation was present in the *right kidney.* In some parts, tubule formation was seen and in others the structure approached that of the clear-celled variant. The bulk of the growth was made up of very large cells with abundant finely granular eosinophilic cytoplasm having large nuclei with prominent deeply eosinophilic nucleoli, often multiple. The size and shape of the tumour cells were very variable, and bizarre forms and tumour giant cells were common. In some places, necrosis and hæmorrhage were present. Mitoses were uncommon. Lymphatics and occasional venules contained growth.

All the *lymph nodes* examined (mediastinal, para-bronchial, cœliac, para-aortic and mesenteric) were almost completely replaced by growth having similar characters to those of the renal tumour. Large masses of growth replaced lung tissue which in some parts was infected. Dilated perivascular and peribronchial lymphatics were full of growth and, very rarely, small blood vessels contained carcinoma cells.

### *The cytoplasmic inclusions*

In the primary tumour, where the cells were not necrotic, in the lung metastases and generally distributed throughout the growth in the lymph nodes were refractile deeply eosinophilic bodies. These were not present in the tumour-free parts of the right kidney nor in any other organ examined. Nearly every malignant cell contained one or more of these inclusions, the number bearing an indirect relation to the size. Even where single cells in remote lymphatics were seen, the cytoplasm contained the bodies. In size, they varied from only just visible granules to large masses up to $25 \times 8\ \mu$. The smallest bodies were round, but the larger ones oval, bean-shaped, horseshoe-shaped, dumb-bell-shaped or in irregular masses. In other characters the bodies were generally similar to those in case 1.

## STAINING REACTIONS OF THE CYTOPLASMIC BODIES

The bodies were investigated by standard histological and histochemical techniques, including methods for the specific demonstration of various intracellular granules.

The bodies gave a negative reaction with the periodic acid-Schiff technique, thus eliminating glycogen, simple and complex mucopolysaccharides, mucoproteins, glycoproteins and glycolipids. Feulgen's reaction was negative. Tests for iron and fat were also negative and under polarised light the bodies were isotropic.

With the general histological stains, the cytoplasmic bodies were uniformly eosinophilic. With Mallory's phosphotungstic acid hæmatoxylin and with iron hæmatoxylins they were blue-grey and blue-black respectively. Kühne's hyaline stain gave only a very faint blue, and Gram's stain was not well retained. With van Gieson's stain the bodies stained a bright yellow.

Of the various stains, carbacid fuchsin (Lendrum, 1945) and Laidlaw's acid fuchsin-orange G were the most specific, the fuchsin staining being wholly confined to the bodies. With carbacid fuchsin the staining was patchy and inconstant, and uniform results were not readily obtained. The bodies did not appear homogeneous and gave an impression, not definitely confirmed by any other method, of a coagulum confined within a limiting pellicle. Phloxin-tartrazine (Lendrum, 1947) gave excellent definition, the bodies being homogeneous and strongly phloxinophilic, retaining the red stain rather longer than the majority of the red cells. In the subsequent investigations, this stain was adopted as a standard.

Inclusion bodies in the renal epithelium of the newborn have been described by various authors, and as material from a recent case (Cappell and McFarlane, 1947) was available, the staining reactions were compared with those of the present tumours. Material from the submaxillary virus disease of guinea-pigs was also employed, as this condition occasionally involves the kidneys, and the cytoplasmic globules of hyaline droplet change in renal epithelium were also studied. No tinctorial or morphological similarity was found between the inclusions in the newborn or in the guinea-pig and those of the present cases. Comparison of the staining reaction of the tumour inclusions with hyaline droplets in renal epithelium showed some tinctorial similarity, particularly with phloxin-tartrazine, but the larger hyaline droplets exhibited somewhat greater refractility and a more strongly phloxinophilic property. Fundamental differences were, however, observed. The hyaline droplets were P.A.S. positive, and also stained strongly with Kühne's hyaline stain and Weigert's fibrin stain, in contrast to the tumour bodies.

### CYTOPLASMIC BODIES IN OTHER RENAL TUMOURS

A specific search for cytoplasmic inclusions was made in a series of renal tumours, since their presence in small numbers in previous cases might have been overlooked in the routine hæmatoxylin and eosin preparations. Accordingly, sections from 70 cases of renal tumour were prepared, stained with phloxin-tartrazine and searched carefully for inclusions.

The series included 58 cases of renal carcinoma, 55 cases of renal adenoma, and 7 renal rests and miscellaneous cases. Fixation was, as before, by corrosive formol or formol saline.

In sixteen of the renal carcinomata, refractile spherical phloxinophil bodies were found in the malignant cells. In all cases, the bodies were scanty and appeared as multiple small spherical globules situated peripherally in large swollen cells, and lying between the nucleus and the free margin, or as large single spherical globules. The inclusions were never located at the base of the cells and were almost invariably spherical. In addition, they were rather more highly refractile, and even more phloxinophilic than those in the two tumours under review. Investigation of the staining reactions of the inclusions in this series shows that they were tinctorially identical with the hyaline droplets in renal epithelium. With carbacid fuchsin, the fuchsin staining was wholly confined to the bodies, which presented a much more homogeneous appearance than did those in cases 1 and 2. With phloxin-tartrazine the bodies were readily identifiable by virtue of their greater refractility, even when their size approximated to that of a red cell, and despite incomplete decolorisation of the red cells. The staining reactions of both large and small inclusions in the series were identical.

### DISCUSSION

Cellular inclusions are not infrequent in malignant tumours, and in the past have given rise to speculation about the possible parasitic and virus ætiology of neoplasms. Various accounts of both cytoplasmic and intranuclear inclusion bodies in tumour cells have been given. The cytoplasmic bodies include the " bird's eye " inclusions of glandular carcinomata, which are characteristically located between the cell nucleus and the alveolar lumen, and contain a central basophilic portion surrounded by a protoplasmic rim or " halo " (Ewing, 1940; Willis, 1948). Cytoplasmic inclusions have been most frequently reported in tumours of glandular origin, and as a rule are the result of disordered attempts at the formation of secretion. In the gliomata, the intranuclear inclusions are compatible with virus activity (Russell, 1932). In other cases, as in the muscle sarcomata, abnormal configurations of the nuclear chromatin may present a superficial resemblance to virus inclusion bodies or to protozoan agents. Prominent acidophil nucleoli are characteristic of rapidly growing cells, both simple and malignant.

In the renal carcinoma of the leopard frog (Lucké, 1934*a* and *b*; 1938*a* and *b*) where the general architecture of the growth corresponds very closely to the papilliform areas of the tumour in case 1, the inclusions are again exclusively intranuclear, being of Cowdry's type "A".

In the present cases, the association of cytoplasmic inclusions with acidophil intranuclear bodies in a regular pattern suggested the activity of a virus. On detailed examination, however, the bodies differed in various fundamental respects from intranuclear and cytoplasmic bodies of known virus origin.

The large eosinophilic nucleoli in the renal tumours bear a close resemblance to the earliest stages of development of the intranuclear inclusions of guinea-pig submaxillary virus disease, but the fully developed virus bodies differ radically from the nucleoli, which show no significant difference from the nucleoli of other renal carcinomata without cytoplasmic inclusions, and from the nucleoli in other carcinomata and similar rapidly growing cells.

The tumours do not arise from glandular epithelium. Although the secretory function of renal tubule cells for certain metabolic products, dyes, etc., is now generally accepted, this activity is not associated with the formation of visible intracellular secretion products. Materials absorbed from a tubular lumen are most abundantly stored between the luminal margin of the tubule cells and the nucleus, but only rarely between the nucleus and the basement membrane. In case 1 the position of the material in the cells is in direct contrast to that of products of secretory activity. Also the subnuclear localisation of the bodies is opposed to the possibility that they have been formed by absorption from the fluid contents of the renal tubules. Moreover, in case 2, the bodies were found in metastases in widely different structures such as lymph nodes and lung. However, the possibility of absorption of some substance from the blood stream remains. It has been observed that the greatest concentration of these bodies is in the peripheral parts of the tumours, and that they appear to undergo lysis with the onset of necrosis. This indicates that the viability of the cells is necessary for their preservation and the presence of the bodies cannot be attributed to degenerative change.

Eosinophilic cytoplasmic inclusions occur in a number of human infections, but these differ fundamentally from the bodies in the present cases, being demonstrably composed of aggregations of elementary bodies.

It is concluded that the bodies in the tumours do not correspond to known virus inclusions. There is no evidence that the material in the cells is a secretion, and degenerative processes are clearly not involved in the production of the inclusions. It seems certain that the bodies do not arise from absorption of extraneous matter. The remaining possibilities are that the cytoplasmic bodies may represent either material absorbed from the blood, which the cells are unable to metabolise, or that the bodies are accumulations of some by-product of the abnormal metabolism of the tumour cells. The greater concentration and greater size of the bodies in the peripheral and most actively growing parts of the tumour in case 1 and in the metastases in case 2 may be taken as consistent with either hypothesis.

A notable feature in case 1 is the absence of inclusion bodies in the secondary growths. In these, all the cells showed features similar to the cells in the primary growth that were in the early stages of nutritional impairment, but it is not clear whether there was any actual impairment of vitality in the secondaries. The presence of a heavy polymorph infiltration in the absence of any demonstrable pyogenic infection, however, may indicate some such disturbance.

## SUMMARY

Two cases of renal carcinoma are described in which the majority of the viable cells contain one or more cytoplasmic inclusions. The morphology and staining reactions of these bodies were compared with those of known virus origin, and with those of hyaline droplets in renal epithelium. In each instance fundamental differences were demonstrated. A series of renal carcinomata was investigated for corresponding structures with negative results.

Our thanks are due to Prof. D. F. Cappell for the use of material from the case of renal inclusion disease, and for his advice and criticism, to Mr W. A. Galbraith and Dr A. Barham Carter for access to the respective case histories, and to Mr H. C. Gray, A.R.P.S., of The Victoria Infirmary, Glasgow, for the Ektachrome photomicrographs.

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# EXPERIMENTAL PULMONARY HÆMOSIDEROSIS

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(Plates CXXVI-CXXIX)

The well known condition of brown induration of the lungs, found particularly in relation to mitral stenosis, consists in part at least of collections of hæmosiderin resulting from hæmorrhage into the lungs. The exact source of the hæmorrhage is uncertain although it has been generally presumed to arise from the congested capillaries in the alveolar walls. Lendrum *et al.* (1950) have asserted that the bleeding occurs from the anastomoses between the bronchial and pulmonary vascular systems in the walls of the respiratory bronchioles on the grounds that the hæmosiderin accumulations are found in close anatomical relationship to the sites of these anastomoses. The purpose of this paper is to describe experiments in rats which show that similar hæmosiderin accumulations can result from blood which is at first diffuse in the lung. These accumulations happen to be near the places where, in the human, the vascular anastomoses are believed to be normally present, and in mitral stenosis to become varicose (Ferguson *et al.*, 1944). The experiments show that the localisation of the hæmosiderin near the sites of anastomoses is probably coincidental and that the conclusions of Lendrum and his co-workers are not necessarily valid.

## Experimental method

Blood was obtained from the inferior vena cava of anæsthetised rats and injected endotracheally into other rats. In earlier experiments untreated blood was used but in later ones it was heparinised so that a number of rats could be injected from a single donor. The addition of heparin did not affect the results.

Endotracheal injection under direct vision is quite simple after a little practice. Under ether anæsthesia, the animal is held supine with the head slightly extended and projecting over the edge of a bench. A pair of non-toothed forceps, held closed, is introduced into the pharynx. The tips are then allowed to part by the spring of the forceps which has to be fairly strong. With the aid of a head mirror the vocal cords can now be seen opening and closing rather rapidly with respiration. A blunt-ended hollow needle (7.8 cm. long) is passed between the cords until the tip is judged to be about at the level of the bifurcation of the trachea. With the needle in place, the rat is held vertical with head uppermost and the required amount of blood injected through the

But even after 9 months the quantity and distribution of this interstitial pigment did not change to any great degree and never became a very striking feature.

## DISCUSSION

The object of these experiments was to consider experimentally the principle that focal collections of hæmosiderin-containing phagocytes, such as those seen in the lungs in cases of mitral stenosis, may be the result of diffuse hæmorrhage within the lung. The experiments have shown clearly that this can occur at least in the lungs of rats. Because of the differences in the minute anatomy of the human and rat lung, a very close comparison of the anatomical distribution of the aggregations in the two species need not be attempted but the similarity in appearance is sufficiently close to warrant the conclusion that the fundamental process is the same in both.

The reason for the accumulation is uncertain. One possibility is lymphatic obstruction. One of the characteristics of the human disease is the change in the interstitial tissue. The walls of the affected alveoli show fibrous thickening, and sometimes the elastic fibres, especially around the blood vessels, become impregnated with iron and provoke a giant-cell reaction in their vicinity (Lendrum, 1950). Lendrum suggests that these proliferative changes obstruct the lymphatic drainage and thus contribute to the formation of the focal accumulations of phagocytes, but the present experiments show that the aggregations occur in the absence of interstitial changes. Such changes have not in fact been observed in the rat's lung. Their appearance may depend upon the time factor because the longest interval in these experiments after the blood has been first administered is just under 12 months, whereas hæmosiderin is probably present in the human lung for a far greater time before the interstitial changes appear. Another possible explanation for this difference is that in mitral stenosis the lung not only contains hæmosiderin but is also in a state of chronic venous congestion. The rat lungs on the other hand are healthy.

There is no obvious explanation how or why these aggregations should be so constant in size and distribution. Lendrum *et al.* (p. 255) believe that "*the size, shape, and distribution of the deposits point definitely to the vessels of the small bronchi as the source of the hæmorrhage*". That this idea of focal hæmorrhage is not necessarily correct is shown by some observations made during these experiments. Some rats died immediately following an endotracheal injection of blood. In these animals the distribution of the blood, although somewhat patchy, involved large, confluent areas of the lung; it did not collect in isolated clusters of alveoli. Within a few days this diffusely distributed blood had been replaced by small discrete aggregations of alveolar phagocytes containing what remained of the injected blood in the form of hæmosiderin. If after numerous previous injections,

# EXPERIMENTAL PULMONARY HÆMOSIDEROSIS

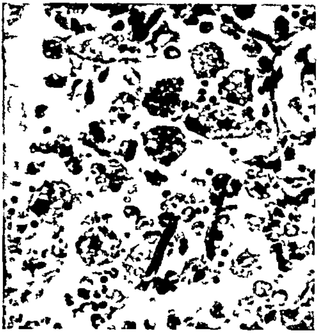

FIG 1—From a rat killed 2 days after a single endotracheal injection of blood Many of the red blood cells have been engulfed by alveolar phagocytes others are still lying free within the alveolar spaces Hæmalum and eosin ×400

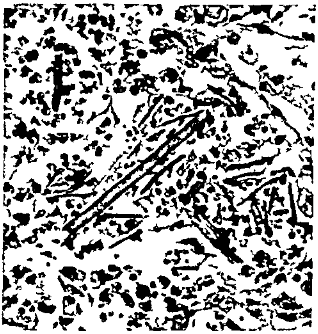

FIG 2—Another field from the same section as fig 1 to show the large extracellular crystals of hæmoglobin H and E ×400

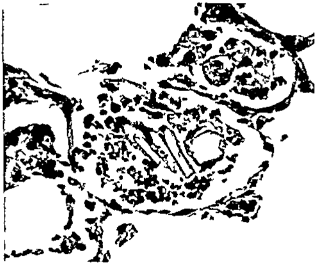

FIG 3—Four days after a single injection of blood The hæmoglobin crystals have become engulfed by large multinucleated phagocytes within the alveolar spaces H and E ×300

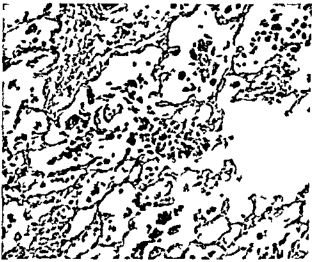

FIG 4—Seven days after a single injection of blood Early aggregation is apparent, some of the alveoli being crowded with phagocytes containing hæmosiderin granules Perls hæmalum and van Gieson ×115

EXPERIMENTAL PULMONARY HÆMOSIDEROSIS

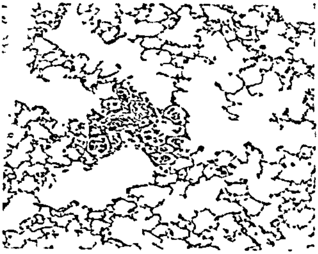

FIG. 5.—Ten days after a single injection of blood. Most of the hæmosiderin-containing phagocytes have collected into a rather loose aggregation. H. and E. ×70.

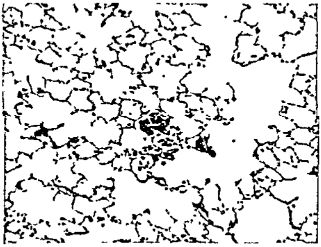

FIG. 6.—Fourteen days after a single injection of blood. The aggregation is compact and well formed; the surrounding alveoli still contain a few phagocytes. Perls, hæmalum and van Gieson. ×75.

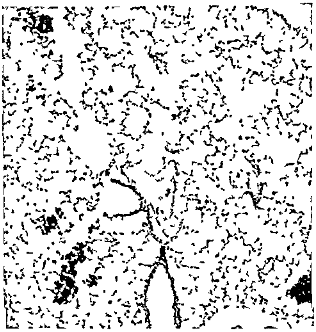

FIG. 7.—From a rat given 15 ml. of blood in 1 ml. doses over a period of 13 weeks and killed 8 days after the last injection. Small clusters of alveoli are packed with hæmosiderin-bearing phagocytes. The neighbouring alveoli are almost free of cells. Perls and neutral red. ×40.

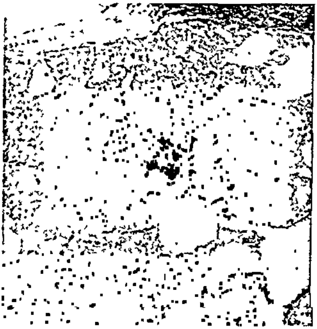

FIG. 8.—From a rat which died immediately following an injection of blood, having had 8 injections previously. A discrete aggregation of phagocytes surrounded by blood diffusely distributed in many of the surrounding alveoli. Perls and neutral red. ×60.

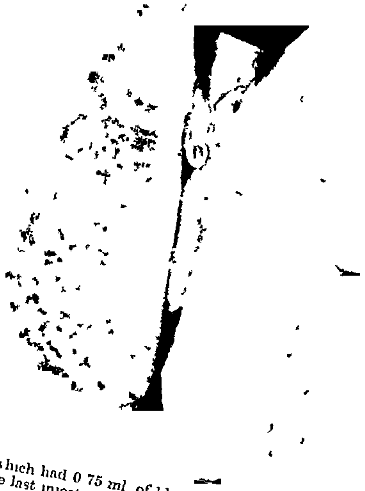

FIG. 9.—From a rat which had 0·75 ml. of blood twice weekly for 12 weeks and died 9 months after the last injection. Discrete spots can be seen beneath the pleura, representing aggregations of hæmosiderin-containing phagocytes. ×2.

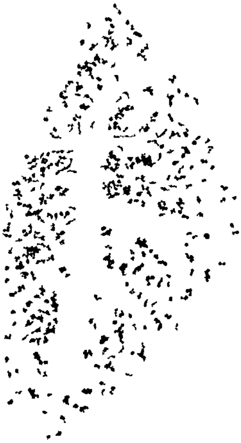

FIG. 10.—An unstained section of a whole lung cut at about 200 μ and mounted on paper by the Gough and Wentworth (1949) technique. From a rat which was given 0·75 ml. of blood twice weekly for 12 weeks and died 4¼ months after the last injection. Aggregations are scattered throughout the lung and are rather more numerous in the dorsal parts. ×2¼.

EXPERIMENTAL PULMONARY HÆMOSIDEROSIS

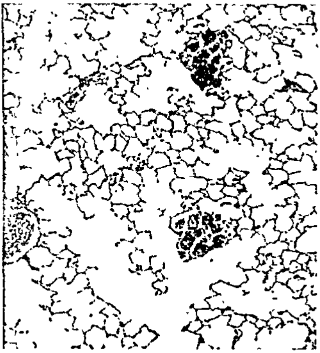

FIG. 11.—From a rat which had 0·75 ml. of blood twice weekly for 12 weeks and died 6 months after the last injection. Two discrete aggregations are included and their relationship to the alveolar ducts can be seen. H. and E. ×45.

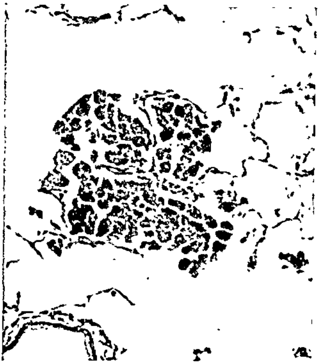

FIG. 12.—From the same rat as fig. 11. The phagocytes are filled with hæmosiderin granules and crowded into a small group of alveoli. From one of the alveoli the phagocytes bulge into the alveolar duct. H. and E. ×180.

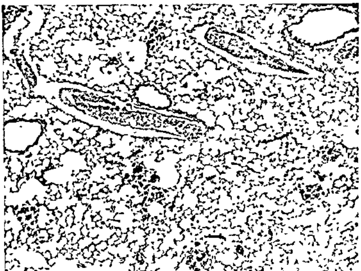

FIG. 13.—From the same rat as fig. 11. Several aggregations are included. The distribution away from the peribronchial and perivascular connective tissue is well shown. H. and E. ×20.

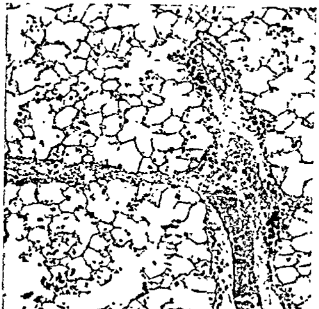

FIG. 14.—From a rat which received 5 weekly injections of 1 ml. of blood and died 4 days later. There is a little hæmosiderin — mainly intracellular — in the perivascular connective tissue. Perls, hæmalum and van Gieson. ×60.

death should immediately follow a further injection, the aggregations of hæmosiderin, surrounded by large areas of alveoli filled with blood, form a very striking picture (fig. 8), emphasising how diffuse the blood is initially and how focal is the blood pigment finally. These findings offer sufficient proof that focal collections of hæmosiderin-containing phagocytes can result from diffuse hæmorrhage into the lung and that they are not necessarily the result of focal hæmorrhages.

If focal hæmorrhage is not the cause of these collections, then what is ? It may be that the lung is able to get rid of most of the blood by ciliary movement, together with the flow of fluid up the bronchial tree, leaving behind small pools of blood which, because of some undiscovered anatomical feature cannot be emptied by such means. The alveoli containing the hæmosiderin-bearing phagocytes might be thought to be backwaters with only a very inefficient and perhaps narrow communication with the alveolar duct. That this is not so is shown by the occurrence of collections of phagocytes actually bulging into the alveolar duct through a widely patent alveolar ostium (fig. 12). The reason for the retention of the phagocytes in this position is quite obscure. Another possibility is that the red blood cells in the alveoli are engulfed and changed into hæmosiderin by the alveolar phagocytes which then migrate by chemotaxis to collect in certain sites in the pulmonary tissue in preparation for an attempt—apparently mainly unsuccessful—to rid the lung of this foreign particulate material. It is also possible that the phagocytes ultimately become paralysed and inert by virtue of their excessive load of hæmosiderin so that they persist at these sites for an indefinite period. The findings in the rats receiving a single injection support to a certain extent this theory of the migration of the phagocytes because, whereas after two days hæmoglobin is seen to be changing into hæmosiderin over fairly large areas of the lungs, after 14 days the hæmosiderin—which is now contained in the alveolar phagocytes, has accumulated in the circumscribed foci described.

Thus while the above experiments have been successful in producing aggregations of hæmosiderin-laden phagocytes very similar to the focal collections seen in the lung in cases of mitral stenosis in the human, they have not led to a precise explanation of the reason for, or the process of, such aggregation. They have, however, shown that these aggregations can be the result of diffuse intra-pulmonary hæmorrhage and that it is not necessary to postulate an origin in focal hæmorrhages from any particular anatomical site.

## SUMMARY

Blood has been injected endotracheally into rats' lungs resulting in the formation of intra-alveolar aggregations of hæmosiderin-containing phagocytes.

These aggregations are similar to those found in the human lung in chronic venous congestion.

This finding suggests that the aggregations in the human lung can follow diffuse hæmorrhage and that they are not necessarily the result of focal hæmorrhages from dilated vascular anastomoses in the walls of the respiratory bronchioles.

It is with pleasure that I record my thanks to Professor Jethro Gough for his help in the preparation of this paper and to Mr J. P. Napper for assistance with the photography.

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576 . 809 . 734 : 576 . 852 . 211

# THE SEROLOGY OF POLYSACCHARIDES FROM THE TUBERCLE BACILLUS

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For over forty years it has been known that crude antigens from the tubercle bacillus will give a positive complement-fixation test with sera from tuberculous humans. Numerous workers (*e.g.* McIntosh and Fildes, 1914 ; Punch, 1920) have used this test and have claimed from 80 to 90 per cent. of positive reactions, but the precise nature of the antigen responsible has not been determined. Schaeffer (1940) reported that serum from patients with tuberculosis contains both anti-polysaccharide and anti-protein antibodies, but his work has not yet been confirmed. To examine this problem I have compared the serological activity of a number of pure tubercle polysaccharides with both animal and human sera, and the present paper reports the results. My experiments show that tubercle polysaccharides react with sera from experimentally infected animals, but that they do not react with sera from patients with tuberculosis.

## Literature

There have been many studies of the chemical nature and serological activity of tubercle polysaccharides The serological activity has usually been assessed with antisera made by injecting animals with heat-killed or chemically-treated bacilli. Rabbit and horse sera have been most frequently employed and positive precipitin reactions have been obtained with each. Tests of this type have been used on material from the bacterial cell (Zinsser and Parker, 1923 ; Laidlaw and Dudley, 1925 ; Heidelberger and Menzel, 1931-32, 1937 ; Chargaff and Schaeffer, 1935-36 ; Menzel and Heidelberger, 1939), from culture filtrates (Mueller, 1926 , Branch, 1931-32 ; Seibert, Pedersen and Tiselius, 1938 ; Seibert, Stacey and Kent, 1949), and from cell lipoids (Anderson, Reeves and Stodola, 1937 ; Anderson, Creighton and Peck, 1940). The presence of anti-polysaccharide antibodies in animals inoculated with living virulent organisms has also been recorded : in rabbits by Seibert *et al.* (1949), in goats by Steenken (1941), and in guinea-pigs by Schaeffer (1940). None of these authors reported any tests with human serum except Seibert *et al.* (1949) who obtained positive precipitin reactions in 2 of 5 sera, but with only one of two samples of a purified polysaccharide. Other reports of positive precipitin reactions with human sera and tubercle polysaccharides have been made—for example, by Kropp and Foley (1944) in 2 of 11 tuberculous sera and in 2 of 5 normal sera, and more recently by Choucroun (1947, 1949) in half of the 298 sera she tested. Precipitin reactions in human tuberculosis appear to be inconsistent and their value is difficult to assess. Early workers (Bezançon and de Serbonnes, 1909 ; Calmette and

Massol, 1910) regarded precipitins in the human disease as unreliable for diagnosis and often not caused by antigen-antibody reactions. They used crude antigens containing proteins and lipoids as well as polysaccharides. Porter (1910) claimed that precipitin reactions obtained with tuberculin and human sera were completely non-specific.

Pinner (1928) compared the serological activity of Zinsser and Parker's (1923) polysaccharide with animal and human serum. A sheep antiserum made by injecting the animal with heat-killed bacilli gave a positive precipitin reaction, but serum from a sheep infected with the living organism was negative. Serum from cases of human tuberculosis was also negative when tested with the complement-fixation test.

## MATERIALS AND METHODS

### *Polysaccharides*

The following were available :

**Fraction 92.** (Seibert *et al.*, 1949). A polyglucosan, containing no pentose or other sugar except glucose.

**Fractions 3 and 4.** Dextro-rotatory sugars as prepared by Haworth, Kent and Stacey (1948). Fractions 3 and 4 were probably similar to each other.

**Fractions 7 and 8.** Sugars with a low dextro-rotation and probably identical with each other (Haworth *et al.*).

**Fraction $T_4B_2$.** Dextro-rotatory material made by ethanol precipitation of a watery extract of tubercle bacilli (A. S. Jones, personal communication).

**Fraction L.P.** Dextro-rotatory material made from an ether extract of heat-killed organisms (A. S. Jones, personal communication).

All the above compounds were made from organisms grown on synthetic media containing no protein or other complex organic materials. The fractions 3, 4 and $T_4B_2$ may be regarded as similar to each other and are probably similar to Heidelberger and Menzel's (1937) C fractions. Fractions 7, 8 and L.P. resemble each other and the B fractions of Heidelberger and Menzel (1937). It is of course impossible to say that complex preparations of this type are quite the same as those made by other workers using different methods of extraction. Their similarity in chemical constitution and their reaction with the same immune serum (see page 737) indicate, however, that they are closely related. All the compounds were shown to be protein-free by the Millon, Biuret and Sakaguchi tests.

### *Antisera*

**A 5807.** An immune horse serum prepared in 1925 by the weekly injection of increasing doses of a defatted human-type tubercle bacillus (Seibert, personal communication). This serum has been used by many American workers (*e.g.* Heidelberger and Menzel, 1931-32, 1937 ; Menzel and Heidelberger. 1939 : Seibert *et al.*, 1938, 1949).

**C 150.** An immune horse serum prepared against heat-killed-bovine type organisms (Schaeffer, 1946) The organisms were grown on Besredka's medium (Schaeffer, personal communication)

These two sera were used because they are high-titre anti-polysaccharide immune horse sera of a type used by other workers

**Rabbit sera** Fifteen rabbits were immunised with living virulent human-type tubercle bacilli, which had been grown on Lowenstein-Jensen medium for five weeks Ten mg of moist growth were given intravenously each week for three weeks, and the animals were bled ten days after the last injection This procedure did not always give a good serum, but if the animal was rested for two or three weeks, and then given a dose of 1 2 mg, a positive serum was usually obtained There were only two failures with this method and the animals tolerated the injections well In a period of eighteen months only two were lost through tuberculosis, and each of these had received a total of 100 mg of virulent organisms in three months The other animals remained healthy

**Guinea-pig sera** These were obtained by stunning and bleeding animals which had been inoculated in the groin two months previously with 0 1 mg of living virulent human-type organisms The tubercle bacilli had been grown on Lowenstein Jensen medium for five weeks Emaciated animals were not used, and all the sera were obtained from animals with well developed local lesions and some spread of the disease to the abdominal viscera

**Human sera.** Blood was collected from patients with moderately advanced pulmonary tuberculosis, from minimal cases and from patients with involvement of the bones or of the urogenital tract as well as of the lung Sera of patients dangerously ill or moribund were not used As a rule each serum was used within three days of its collection For some of the experiments with homologous complement (see below) the sera were preserved with buffered hypertonic saline containing sodium azide as recommended by Richardson (quoted by Mackie and McCartney, 1948) Ten sera were tested against each preparation and in all 200 sera were collected Sera from Mantoux-positive and from Mantoux-negative healthy people were used as controls

### *Precipitin tests*

All the polysaccharide fractions dissolved readily in water and in isotonic saline As a preliminary to each test a range of antigen dilutions was set up against a constant volume of serum, which was itself diluted 1 in 2, and the mixture was incubated at 37° C for two hours and then left in the refrigerator overnight at 2 4° C For precipitin-absorption tests, the equivalence zone was first approximately determined and the serum was then precipitated in this zone After being left overnight in the refrigerator, the mixture was centrifuged, and the supernatant fluid was removed and treated with one-tenth of the volume of polysaccharide solution originally added. It was then left in the refrigerator for 48 hours, after which it was again centrifuged. If any precipitate appeared, another volume of polysaccharide was added, and the process was repeated. The absorbed serum finally had a small excess of antigen as was shown by testing a sample with more serum.

## RESULTS

The highest titres obtained with the animal sera (table) indicate that the serological activity of these polysaccharides was approximately equal to that recorded by other workers. The results of the precipitin-absorption tests are not given, because all were positive, *i.e.* each of the compounds was capable of absorbing the antibody for any one of the others. The human serum did not give precipitin reactions, with the exception of two sera which produced fine precipitates with fractions 8 and L.P. and these results could not be obtained consistently.

TABLE

*Precipitin reactions of tubercle polysaccharides with immune animal sera. The figures refer to antigen dilutions*

| Polysaccharide fraction | Optical activity (degrees of dextro-rotation) | Highest titre with serum | | | |
|---|---|---|---|---|---|
| | | A 5807 (Horse) | C 150 (Horse) | Rabbit | Guinea-pig |
| 92 | 165 | 100,000 | 300,000 | 20,000 | 100,000 |
| 3 | 80 | 1,000,000 | 1,000,000 | 50,000 | 10,000 |
| 4 | 85 | 1,000,000 | 1,000,000 | 10,000 | 50,000 |
| 7 | 25 | 500,000 | 500,000 | 200,000 | 1,000 |
| 8 | 28 | 1,000,000 | 1,000,000 | 10,000 | 2,000 |
| L.P. | 25 | 1,000,000 | 1,000,000 | 50,000 | 4,000 |
| $T_4B_2$ | 75 | 50,000 | 500,000 | 25,000 | 1,000 |

Similar, but not identical, results to those in the last two columns were obtained with other rabbit and guinea-pig sera.

### *Additional tests on human sera*

As the precipitin tests were negative, two other methods of detecting antigen-antibody reactions were tried: (1) complement fixation; and (2) agglutination.

*Complement fixation* was attempted by two methods. In the first the sera were inactivated by heating for 30 minutes at 56° C. and a constant amount of the serum was then allowed to react with a range of dilutions of the polysaccharide in the presence of 2 M.H.D. of guinea-pig complement. In the second method the human complement in the freshly drawn serum was used in the hope that the homologous complement would be more efficiently fixed by human antibody.

To do this, dilutions of fresh unheated serum were incubated with a constant quantity of polysaccharide, and the amount of complement remaining was estimated and compared with the values obtained from a similar series of serum dilutions, which had been incubated with isotonic saline. Any difference between the two titres would represent fixation. Sera from Mantoux-negative people were treated in the same way as controls. All these tests gave negative results.

*Agglutination tests.* Several methods of adsorbing antigens to serologically neutral particles were tried. Collodion particles were made and used as recommended by Cannon and Marshall (1940), but these proved unreliable and gave inconstant results. Carmine particles, as used by Jones (1945), and suspensions of *Serratia marcescens* (Roberts and Jones, 1941) were also unsuccessful, as were suspensions of *Cl. welchii* from which the material reacting positively in Gram's method had been removed by the method of Henry and Stacey (1946). Another attempt was made using sheep red corpuscles for adsorption of the antigen (Burnet and Anderson, 1946 ; Middlebrook and Dubos, 1948) and suponsions were also made of the polymer, polystyrene, which was precipitated from solution in ethyl acetate by acetone and water. These last two methods were used on fractions 4 and 8 only ; they also gave negative results. Since all these tests were negative the conclusion was reached that with the methods used the purified polysaccharides specified did not react with sera from tuberculous humans.

## DISCUSSION

The results obtained with animal sera support the conclusions of other workers : immune horse sera made with heat-killed or chemically-treated organisms have anti-polysaccharide antibodies in high titre, while rabbits and guinea-pigs inoculated with living virulent human-type organisms have similar antibodies but in lower titre. My experiments also show that infected humans do not possess detectable antibodies to protein-free polysaccharides. It appears, therefore, that the predominant antibody in the sera of tuberculous humans does not react with the same determinant group as the antibody in experimentally-infected animals. Such animal sera are probably polyvalent and contain anti-protein as well as anti-polysaccharide antibody. They may therefore have an antibody with the same specificity as that present in tuberculous humans, but this is not identical with the high-titre anti-polysaccharide antibody. The chemical nature of the specific radicle reacting with human serum is unknown ; but in a paper * given to the Fifth International Congress for Microbiology held in 1950 at Rio de Janeiro, Iland, Baldwin, Gilbert and Jones claimed that it is associated with a purified protein fraction which has also the power to produce a typical tuberculin-type skin reaction in

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* This paper will be published in the Proceedings of the Congress.

# SHORT ARTICLES

616 . 11—006 . 2—053 . 31

## AN INTRAPERICARDIAL TERATOMA IN A NEWBORN INFANT

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(PLATES CXXX AND CXXXI)

### *Case report*

*History.* Baby M., a female infant, was the first of twins born at the Western General Hospital, Edinburgh, on 17th March 1950. She weighed 3 lb. 10 oz. at birth and only lived for 15 minutes. She was very œdematous and cyanotic at birth and failed to respond to resuscitation. The mother was healthy and aged 36 years and had had eight previous children, the last two being twins born in April 1949. All the previous children were alive and well. She had felt quite well during her recent pregnancy but had a considerable degree of hydramnios and some œdema of the legs for 3 weeks prior to admission. Her blood pressure was normal. The Wassermann reaction was negative and her blood was group O, Rh positive. She was admitted to hospital on account of sudden onset of labour at the 30th week of pregnancy. She was delivered spontaneously of twins on the 17th March 1950 after a normal labour. There was a single placenta with one chorion and two amniotic sacs. The placenta was complete and was not infarcted. The second twin, also a female, failed to thrive and died after 22 days of extra-uterine life. Necropsy did not reveal any organic disease. The heart and pericardium were normal.

*Necropsy.* The body was that of a small female infant weighing 3 lb. 10 oz. The infant was extremely hydropic, the abdomen was grossly distended with fluid and the subcutaneous tissues were very œdematous.

There was 30 c.c. of clear straw-coloured fluid in each pleural sac and the lungs were collapsed. The pericardial sac was greatly distended and contained about 20 c.c. of clear fluid. The heart was very small, measuring 2·5 cm. from base to apex and 2·4 cm. transversely, and was displaced to the right. It was rotated so that the right ventricle occupied the entire anterior aspect of the heart. The cause of this displacement was a large round lobulated tumour measuring 4·4 cm.×3·1 cm.×2·0 cm., which was firmly attached by a pedicle to the left and posterior aspects of the pulmonary artery and ascending aorta, upper aspect of left ventricle and lateral aspect of left atrium (fig. 1). The blood supply of the tumour was derived from several small vessels coming from the adventitia of the aorta.

The chambers of the heart were very small and the foramen ovale was patent but this was not abnormal for the stage of development. The pulmonary valves, pulmonary trunk and main pulmonary arteries were normal. The ductus arteriosus was widely patent. The tricuspid orifice and right ventricle were small but structurally normal. The right ventricle occupied the anterior aspect of the heart. The left ventricle was rotated posteriorly and was also small. The mitral orifice was guarded by minute but structurally normal cusps. The aortic orifice was normal but the first part of the aorta was slightly narrowed as a result of compression by the tumour. The left atrium was small and very flattened antero-posteriorly. There was no left auricular appendix and its position was occupied by the attachment of the pedicle of the tumour mass.

After fixation the tumour was incised. It consisted of multiple cystic spaces with solid tissue in the centre of a number of the larger cavities (fig. 2). The solid tissue was soft and hæmorrhagic. There was little stroma between the cysts which varied in size from a few millimetres to 2 cm. in diameter, and contained clear sticky fluid. There was only a very narrow partition between the wall of the aorta and the cyst cavity. The capsule of the tumour was intimately associated with the adventitia of the aorta and of the pulmonary artery.

*Histological examination.* The tumour was composed of numerous large cysts with thin fibrous walls lined by flattened cuboidal epithelium and separated from one another by varying amounts of solid tissue. The intercystic stroma consisted of fairly vascular myxomatous tissue in which a great variety of structures were identified. Numerous small cystic structures lined by *respiratory epithelium* of tall columnar type were found. Closely associated with the latter were groups of acini of the *salivary gland* type (fig. 3). Some arrangements of acini and ducts resembled pancreatic tissue. In other areas there were masses of undifferentiated *neuroglia* and numerous circular or elliptical spaces surrounded by masses of darkly staining *ependymal cells* were found (fig. 4). In one section a highly vascular connective tissue stroma was covered by a layer of well-differentiated ependymal cells to form a small *choroid plexus.*

Buried in the centre of a mass of myxomatous tissue were several small strips of rather poorly differentiated *squamous epithelium.* At the periphery of one section there was a small circular block of *cartilage.* No striated muscle was found and there was no evidence of the formation of intestinal mucous membrane.

The other organs showed signs of chronic venous congestion.

## *Discussion*

Histological examination showed that the tumour was a benign congenital polycystic teratoma. Respiratory-type epithelium or neuroglial tissue formed the solid part of the tumour and the remainder consisted of mucus-filled cystic spaces.

The tumour had arisen near the great vessels of the base of the heart and thus closely resembles the previous cases reported by Joël (1890), Grimm (1927), Jellen and Fisher (1936), Gebauer (1942-43) and Willis (1946). Indeed the tumour is almost a mirror image of the one reported by Willis. The latter also was closely associated with the aorta and pulmonary artery but was lying on the right side of the heart whereas the tumour in this case was on the opposite side. The tumour reported by Jellen and Fisher also lay along the right border of the heart.

The site of the tumour and its close relationship to the aorta bear out the theory of Willis that an intrapericardial teratoma is a variety of mediastinal teratoma in an unusual situation. All cases of intrapericardial teratoma reported up to the present have been found in close relationship to the great vessels. This close association is of surgical importance as the aorta may be damaged while the tumour is being removed. Such an injury caused a fatal hæmorrhage in the case reported by Gebauer.

The general effects of the teratoma here reported were confined to severe cardiovascular upset in the systemic circulation. The infant died shortly after

INTRAPERICARDIAL TERATOMA

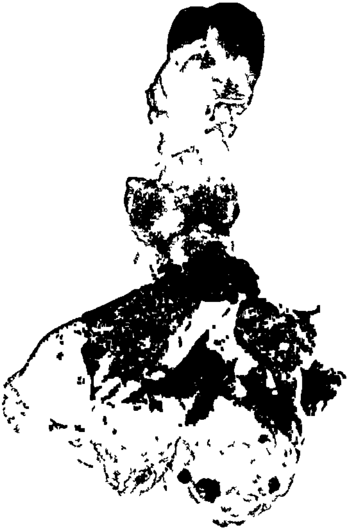

FIG 1—Anterior view of tumour mass showing attachment to the left border of the heart Numerous small cysts protrude from the surface of the tumour

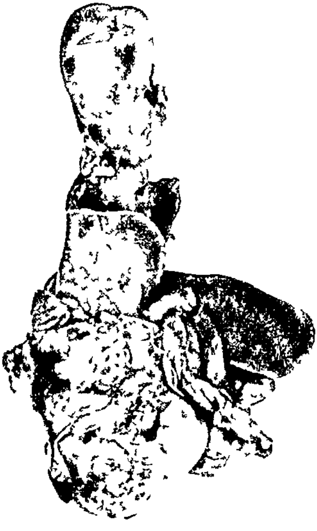

FIG 2—Posterior view of the tumour after incision There is a large thin walled cyst near the lower pole

INTRAPERICARDIAL TERATOMA

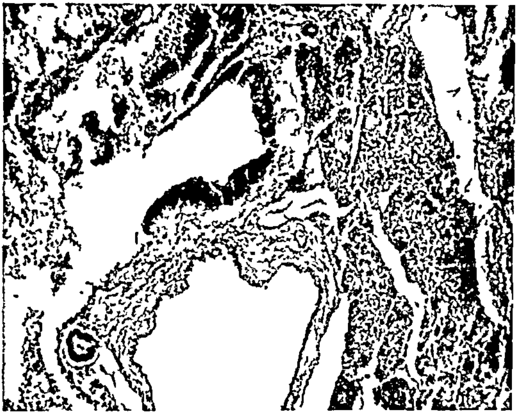

FIG 3—Small cystic space lined by respiratory type epithelium Salivary gland epithelium is present to the right of the cyst and ependymal cell masses are above it Hæmatoxylin and eosin ×75

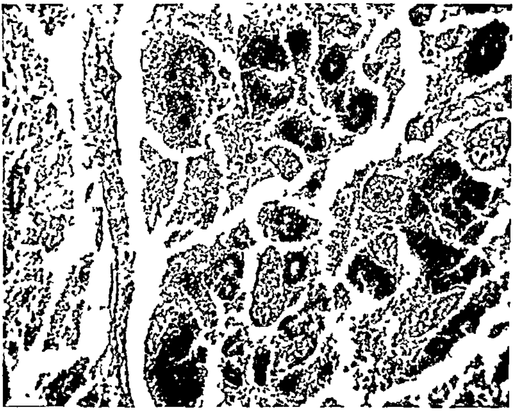

*FIG 4—Masses of ependymal cells and primitive neuroglia with numerous small blood vessels* Hæmatoxylin and eosin ×75

birth and there was no time for changes to occur in the pulmonary circulation such as were found by Willis. The teratoma had caused considerable compression of the heart but had not yet exerted any pressure on the lungs as had the tumour reported by Jellen and Fisher.

All the intrapericardial teratomas so far reported have occurred in infants or children. The present patient was the youngest and the ages of the others varied from 4 weeks (Jellen and Fisher) to 14 years (Joel) Willis's patient was 10 weeks old at the time of admission to hospital. The age of survival depends on the interference by the tumour with the efficiency of the heart. In the present case this interference was so extreme that cardiac failure developed *in utero* and the infant was hydropic at birth In other cases reported, pulmonary embarrassment due to direct pressure of the tumour on the lungs had occurred before the onset of cardiac failure and in such patients the presenting symptoms were cough, dyspnœa and cyanosis

Congenital heart disease may co-exist with intrapericardial teratoma. In the case reported by Jellen and Fisher the foramen ovale and ductus arteriosus were widely patent at the age of 4 weeks These structures were also patent in the present case, but this is normal for the stage of development.

### *Summary*

At autopsy an intrapericardial teratoma was found attached to the heart and great vessels of a newborn infant. This is the sixth case of this type to be reported.

I wish to thank Dr A. Duncan of the Western General Hospital Maternity Unit for the clinical details, and for permission to publish the case. The photomicrographs were made by Mr T. C. Dodds of the Photomicrography Unit of Edinburgh University.

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616 . 212 . 1—022 . 362 : 576 . 851 . 252

# NASAL CARRIERS OF PATHOGENIC STAPHYLOCOCCI IN RELATION TO PENICILLIN SENSITIVITY

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Examination of nasal swabs taken from 126 patients, 47 males and 79 females, on their first attendance at hospital gave the following results :—

Fifty four (42 8 per cent.) harboured coagulase-positive staphylococci, the carrier rate among females was 53 2 per cent. as against 25 5 per cent. among males All 54 strains of *Staph. pyogenes* were penicillin-sensitive. Fifty were alpha-lysinogenic. Phage typing, using the method of Wilson and Atkinson (1945), showed that no particular phage type predominated among the strains of *Staph pyogenes* isolated.

### *Discussion*

It is probable that the high incidence of penicillin-resistant *Staph. pyogenes* found among hospital patients is largely due to cross infection (see Barber and Whitehead, 1949; Barber, Hayhoe and Whitehead, 1949, and Rountree and Thomson, 1949). The incidence of infection with such organisms in the community at large, although more difficult to determine, is almost certainly lower. The authors quoted showed that the incidence of nasal carriers of penicillin-resistant *Staph. pyogenes* is much lower among non-hospital workers than among hospital workers. The present investigation is in agreement with this: although 54 out of 126 patients were found to be nasal carriers of *Staph. pyogenes* on their first attendance at hospital in no case was a penicillin-resistant strain isolated.

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616 . 831—003 . 98—018

## ABSENCE OF BETZ CELLS IN DIFFUSE GLIOSIS OF THE BRAIN WITHOUT DEMYELINATION

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(Plate CXXXII)

Diffuse gliosis of the white matter with little or no loss of myelin was found in 4 cases of microcephaly described by Freeman (1927). This gliosis was most marked in the "lower parts" of the brains. The brains of 22 other mental defectives belonging to different clinical groups have been examined by Meyer and Cook (1937), who were impressed with the frequency of such diffuse gliosis in their material. They confirmed that it was not associated with a loss of myelin and that signs suggestive of preceding or active destructive processes were absent. In a later paper, Meyer and Jones (1939) found similar gliosis in 10 out of 15 cases of mongolism. The gliosis must be distinguished from that seen in Schilder's disease, where it is accompanied by a marked loss of myelin and by a large number of fat-laden phagocytes.

Nothing is known about the ætiology or the pathogenesis of the condition, although Meyer and Cook suggested that some disturbance in the blood supply or defective tissue oxygenation might play a part.

The object of this report is to present a case in which diffuse gliosis of the white matter was associated with an absence of Betz cells. Total absence of Betz cells in a brain with an otherwise normal cortical lamination has not been described before. In a case contributed by Fattovich (1950), absence of Betz cells was found in the brain of an idiot and was associated with numerous cyto-architectonic defects and with certain other features suggestive of tuberous sclerosis.

# DIFFUSE GLIOSIS OF BRAIN

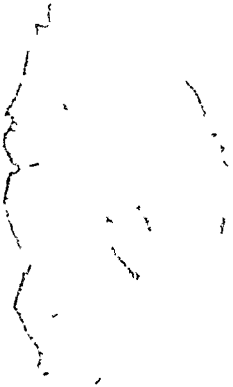

FIG 1 — Coronal section of occipital lobe showing diffuse gliosis of white matter Holzer stain

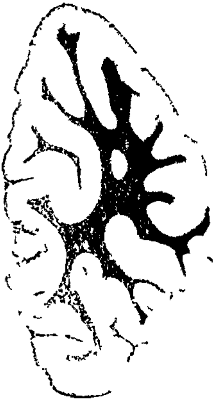

FIG 2 — Coronal section of occipital lo No defect in myelination Heidenhain's myelin stain

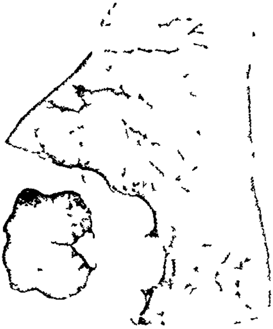

FIG 3 — Fibrous gliosis of medulla and white matter of cerebellum The dentate nucleus and cranial nerve nuclei are spared Holzer stain

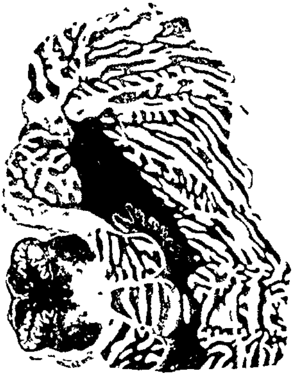

FIG 4 — Medulla and cerebellum with no loss of myelination Heidenhain's myelin stain

## CASE REPORT

### *Clinical history*

The patient was admitted to the Fountain Hospital as a mental defective at the age of 14 months His family history was negative, and his one elder brother, aged 5, was healthy The patient was born after a normal pregnancy and labour, and no jaundice or any other abnormality had been noticed during the neonatal period At the age of 3 months he developed gastro enteritis, for which he was treated in hospital Another period of hospital treatment followed at 1 year, when he had whooping cough His mental backwardness does not appear to have been noticed till he was 9 months old, when he was described as being helpless, and unable to sit or to hold up his head Convulsive fits were said to have occurred at various times prior to his admission to the Fountain Hospital, but none was observed whilst he remained there as an in patient He had, however, small choreiform movements of his face and legs He could not chew his food, had to be fed with a spoon, and his mental level was that of an idiot Whilst in hospital he developed a left otitis media, from which he recovered At the age of 2 years he contracted pneumonia and died

### *Post mortem examination*

The short available autopsy report reads as follows " At post mortem both bases of the lungs showed consolidation There was a superficial engorgement of the brain, and the pyramids of the kidneys showed yellow linear striation due to a deposit of sulphadiazine "

### *Examination of the brain*

The brain weighed 860 g and presented no naked eye abnormality

Examination of sections stained for fibrous glia with the Holzer method showed generalised gliosis of the white matter throughout the brain The gliosis was seen in the cerebral hemispheres, the internal and external capsules, in the periventricular white matter, Ammon's horn cerebellum and in the brain stem (figs 1 and 3) It was more marked in the infra tentorial parts of the brain, and was particularly dense around the aqueduct and in the floor of the fourth ventricle The contrast between the affected white and the intact grey matter was very striking in the cerebellum, where the cortex and the dentate nuclei were spared, and in the medulla, where the inferior olives and the cranial nerve nuclei were likewise unaffected There was no evidence of defective myelination in any part of the brain (figs 2 and 4)

No Betz cells were found in the precentral area although they were searched for in serial sections of the entire cortex containing the central fissure The lamination of the motor area was, like that of the rest of the cortex, normal, and large pyramidal cells were present in its fifth layer None of them was, however, large enough to be described as Betz cells Nissl staining showed an increase in the number and pleomorphism of glial cells in the gliosed white matter Some " Gliarasen " forms were also present The pyramidal tracts were normal

### *Discussion*

It is known that such gliosis as has been described above occurs in mental defectives of different clinical groups so that it is unlikely to be the result of one etiological factor No explanation is offered for the absence of the Betz cells but as there was no gliosis of the motor cortex, their absence must be regarded as congenital

It is now generally agreed that the Betz cells give origin to only a small proportion of the fibres in the pyramidal tract. Several German workers, including Bielschowsky (1917-18), have published cases in which considerable cortical abnormality was associated with intact pyramidal tracts. The preservation of the pyramidal tract in the present case therefore calls for no further comment.

The difficulties of identifying Betz cells are well known and the problem has been fully discussed by Walshe (1942). It is not denied that some of the large pyramidal cells might be identical with the smaller Betz cells and no conclusions are drawn about the relationship between the clinical features of the case and the absence of the larger cells.

## *Summary*

Diffuse gliosis of the white matter without demyelination, or evidence of destructive processes, in the brain of an idiot child was associated with a congenital absence of Betz cells.

My thanks are due to Professor A. Meyer, Dr R. M. Norman and Dr T. Maclardy for their assistance and helpful criticism.

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578. 67

# THE CUTTING OF SECTIONS FOR ELECTRON MICROSCOPY, USING ESTER WAX AS AN EMBEDDING MEDIUM

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(Plate CXXXIII)

Various techniques have been described for cutting very thin sections for the electron microscope. Pease and Baker (1948) and Hillier and Gettner (1950*a* and *b*) have successfully sectioned material doubly embedded in celloidin and hard paraffin wax. Newman, Borysko and Swerdlow (1949) described a method whereby tissue, after dehydration in alcohol, was soaked in the monomer of butyl methacrylate, which was then polymerised, leaving the tissue embedded in a block of solid resin. This method has been successfully used by Wyckoff and his colleagues (Black, Morgan and Wyckoff, 1950; Smith and Wyckoff, 1950) and by Bang (1950). A third method used by Claude and Fullam (1946) requires that material embedded in a mixture of naphthalene be cut at the speed of sound.

We have experimented with the first two methods. We used for the methacrylate-embedded tissue an M.S.E. rotary microtome, advancing the specimen by the thermal expansion of a brass block between the specimen and microtome. Although sections sufficiently thin in places were readily obtained, they were not of uniform thickness and the thickness varied from area to area within each section Furthermore, for the electron microscope to reveal useful histological detail in such a specimen, it is necessary to dissolve away the embedding material, and, as Hillier and Gettner (1950 51) have shown, gross artefacts are thereby caused It was obvious from some of our micrographs that this had taken place

Hoping to improve on our results, we experimented with other embedding methods

(1) Double embedding with celloidin and paraffin wax (M P 56°)
(2) Double embedding with celloidin and paraffin wax (M P 65°)
(3) Double embedding with celloidin and ester wax containing 1 per cent D C 550 silicone *
(4) Embedding with ester wax containing 1 per cent D C. 550 silicone

Ester wax is less hydrophobic than paraffin wax (Steedman, 1947) The addition of the silicone made it less easily wetted and did not change its cutting properties For soft organs method (4) was most successful Pieces, 1 mm cube, of mouse liver or kidney were fixed in 1 per cent osmic acid for 2 4 hours at 4° C , washed, cleared, and dehydrated in three changes of ethyl " cellosolve " (ethylene glycol mono ethyl ether) and infiltrated with ester wax, to which 1 per cent silicone had been added, in a vacuum oven at 56° for 4 hours or over night The tissue and wax were then cast in blocks as described by Steedman The shrinkage and distortion in sections obtained from tissue so embedded were less than by any other method we have tried

Sections were cut using a Cambridge rocking microtome designed to cut at 0 2 $\mu$ A perspex trough, mounted on the knife on the side away from the specimen block, was filled with distilled water so that the sections floated on the surface as they came off the knife edge In this way ribbons of up to 20 sections were readily obtained Although good sections could be obtained at room temperature (*c* 20° C ), better results were obtained by cutting in the cold room at 4° C , there was less tendency at 4° for sections to be split by minor irregularities of the knife edge The block of tissue was brought very slowly across the knife edge Variations in speed produced irregularities in thickness of the sections but with care the variations, as shown by compression of the sections, were less than ±10 per cent

It was of great assistance to watch the cutting of the section through a binocular dissecting microscope having a magnification of about ×20 , the small lens supplied with the microtome was not adequate The meniscus of the water should come up to the edge of the knife , the level of the water may be very slightly lower Sections cut with the meniscus just below the edge were crumpled , given a few seconds, they usually flattened out but always contained some small residual folds which were often not noticed until the specimen was in the microscope, although the folds did not interfere with observation of the areas between them

We found the chorio allantoic and amniotic membranes of the chick embryo difficult to cut when embedded by method (4) The section was apt to split at the junction of the wax and the tissue, and also the tissue in the face of the block tended to become wet, despite the addition of the silicone Satisfactory sections were obtained after embedding by methods (2) and (3) using a final concentration of 8 per cent " necoloidine " (B D H ) When cutting blocks embedded by method (3), however, no addition of alcohol to the water in the perspex trough attached to the knife was found necessary It is probable that much of the difficulty was due to the presence of the erythrocytes of the chick embryo, whose nuclei are rather hard, tending to cause tears in the section where many of them were present, as in branches of the allantoic veins

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* A proprietary name for Silicon oils, the property of Dow Corning Co Inc , U S A

Diethylene glycol distearate alone, with the addition of 1 per cent. silicone, gave better results than ester wax with hard tissues. Ribbons of sections at 0·2 μ were easily obtained even with amnion from developing eggs, although there was still considerable deformation due to compression of the tissue.

For obtaining satisfactory sections, the sharpness of the knife was a limiting factor—the sharper the knife, the thinner the coherent sections which could be produced. The knife was sharpened on a glass hone which had its surface very lightly ground. For sharpening a knife with a very poor edge, the surface of the hone was first dressed with "Sira" abrasive (supplied by the United Kingdom Optical Co. Ltd.) mixed to a paste with thin paraffin oil. As the honing proceeded, the grains of alumina were broken down and the paraffin evaporated, leaving the surface of the hone smooth and dry. The irregularities in the edge after this treatment were about 10-15 μ deep. The hone was carefully cleaned and next dressed with a similar paste of levigated alumina (supplied by the Cambridge Instrument Co. Ltd.). The edge after this treatment contained irregularities about 3-5 μ deep, and the facets in contact with the hone had a good polish. The hone was again cleaned with xylol and dressed this time with a paste of thin paraffin and "Microid" grade 3/50 polishing alumina (made by Griffin and Tatlock Ltd.). After this, the irregularities in the edge were 1-2 μ deep at the most; stretches of edge up to 1 mm. long were obtained with irregularity less than 1 μ deep. The facets of the knife had a brilliant polish and were free of all but the most minute scratches. No further improvement was obtained by the use of the "Gamma" microid alumina, claimed by the makers to be finer than the 3/50 grade. No stropping was necessary after the knife had been so sharpened. Once it had been brought to this state, a few minutes' treatment with the 3/50 grade of alumina was sufficient to restore its edge after it had been dulled by cutting sections. Safety razor blades were not sharp enough or rigid enough to give uniform sections; in fact, it was found advantageous to increase the angle subtended at the edge of the knife by attaching a "back", such as is used in sharpening wedge-shaped knives. This had the effect of increasing the rigidity of the edge and enabled more uniformly thick sections to be cut. The knives used with the microtome were the "light" type, manufactured by the Wilkinson Sword Co. Ltd.; they are slightly hollow-ground on each side.

The sections were picked up on electron microscope grids previously coated with a "Formvar" (polyvinyl formaldehyde plastic) film made by evaporating a 0·5 per cent. solution of Formvar in ethylene dichloride on a glass slide and stripping off the membrane thus formed in a dish of water.

Hillier and Gettner (1950*a* and *b*) recommended that the sections should be put into the electron microscope without removal of the wax; they used the heat generated in the specimen by the electron beam to cause the wax to sublime off in the vacuum within the microscope, but we found that the embedding material, whether ester wax or paraffin wax, was incompletely removed in this way. Other workers appear to have encountered this trouble also (I. M. Dawson, personal communication). We were able to remove the ester wax completely by exposing the grids bearing the sections to radiant heat from hot wires within the vacuum produced by a rotary pump, adjusting the heat so that the wax was just not melted, this latter point being watched by means of the dissecting binocular microscope. Sections treated in this way stood up well to bombardment by the electron beam in the electron microscope.

A few sections were "shadowed" with a 50 per cent. gold-"minalpha"* alloy after the wax had been sublimed off; no increase in definition was found due to the shadowing, although an apparent increase in contrast was produced (fig. 3), while the evaporation of a slightly thicker layer of metal on to the specimen

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* A proprietary name, the property of Johnson Matthey & Co. Ltd., London.